PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 15/82, 15/54, 5/10 A01H 5/00

A1

(11) International Publication Number: WO 91/19806

(43) International Publication Date: 26 December 1991 (26.12.91)

(21) International Application Number: PCT/US91/04036

(22) International Filing Date: 7 June 1991 (07.06.91)

(30) Priority data:
539,763 18 June 1990 (18.06.90) US
709,663 7 June 1991 (07.06.91) US

(71) Applicant: MONSANTO COMPANY [US/US]; 800 North Lindbergh Boulevard, St. Louis, MO 63167 (US).

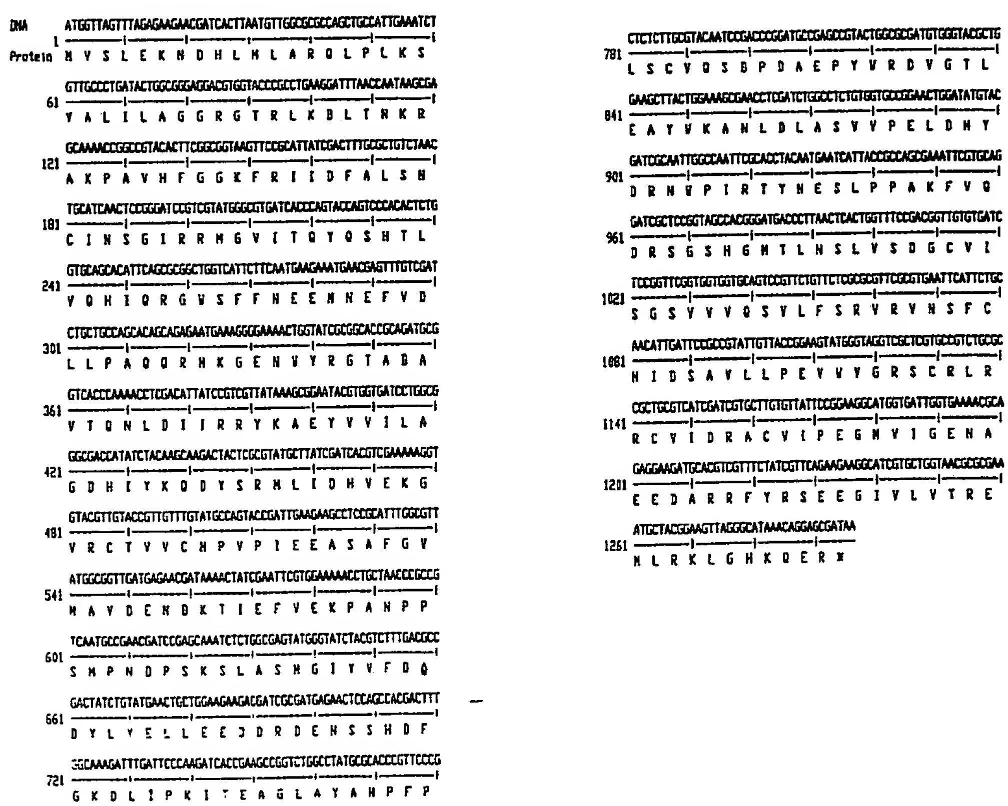
(72) Inventor: KISHORE, Ganesh, Murthy ; 15354 Grantley Drive, Chesterfield, MO 63017 (US).

(74) Agent: BOLDING, James, Clifton; Monsanto Company, 800 North Lindbergh Boulevard, St. Louis, MO 63167 (US).

(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FI, FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), NO, SE (European patent), SU.

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: INCREASED STARCH CONTENT IN PLANTS



(57) Abstract

Transformed plant cells which have increased starch content are disclosed. Also disclosed are whole plants comprising plant cells which express CTP/ADP glucose pyrophosphorylase genes.

BLANK PAGE

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland			SN	Senegal
CI	Côte d'Ivoire	KR	Republic of Korea	SU	Soviet Union
CM	Cameroon	LI	Liechtenstein	TD	Chad
CS	Czechoslovakia	LK	Sri Lanka	TG	Togo
DE	Germany	LU	Luxembourg	US	United States of America
DK	Denmark	MC	Monaco		

BLANK PAGE

INCREASED STARCH CONTENT IN PLANTS

This is a continutation-in-part of a co-pending U.S. application having serial No. 07/539,763, filed on June 18, 1990 and entitled "Increased Starch Content in Plants".

Recent advances in genetic engineering have provided the requisite tools to transform plants to contain foreign genes. It is now possible to produce plants which have unique characteristics of agronomic and crop processing importance. Certainly, one such advantageous trait is enhanced starch content and quality in various crop plants.

Starch is a polysaccharide primarily composed of glucose units connected by alpha 1-4 and alpha 1-6 linkages. It is found in plant cells as water-insoluble grains or granules. During photosynthesis, starch is produced and stored in chloroplasts. Starch is also synthesized in roots and storage organs such as tubers and seeds. In these non-photosynthetic tissues, the starch is found in a form of plastids called amyloplasts. As in the chloroplasts, starch is stored in the amyloplasts as starch granules. The size of the granules varies depending on the plant species.

Starch is actually composed of amylose and amylopectin, two distinct types of glucose polymers. Amylose is composed primarily of linear chains of alpha 1-4 linked glucose molecules. On average, amylose has a chain length of about 1000 glucose molecules. Amylopectin contains shorter chains linked together with alpha 1-6 linkages. On average, amylopectin has a chain length of about 20-25 glucose molecules.

Until recently, there was controversy in the literature as to whether ADPglucose or UDPglucose was the substrate for starch synthesis. With the isolation of *Arabidopsis* mutants lacking ADPglucose pyrophosphorylase it is now accepted that

plants use ADPglucose as the substrate for starch synthesis. There are three steps in the synthesis of starch. All these reactions take place within the chloroplasts or amyloplasts. In the first step, ADPglucose is produced from glucose-1-phosphata and ATP by ADPglucose pyrophosphorylase (EC 2.7.7.27). In the second step, ADPglucose is used by starch synthase (EC 2.4.1.21) to form linear chains of starch containing the α, 1-4 linkage. In the third step, the branching enzyme(s) (EC 2.4.1.18) introduce alpha 1-6 linkages to produce the amylopectin molecule.

The controlling step in the synthesis of starch in plants has been a topic of dispute. Although synthesis of ADPglucose by ADPglucose pyrophosphorylase has been proposed to be the controlling step in starch biosynthesis, this has not been proved. In fact, European Patent Application publication number 0368506 A2, which concerns ADPglucose pyrophosphorylase, questions the role of the enzyme as the rate limiting step in starch biosynthesis. An argument against ADPglucose pyrophosphorylase being the controlling enzyme can be made from the results with an *Arabidopsis* mutant (Lin, 1988a,b). This mutant, TL46, was found to contain only about 5% of the ADPglucose pyrophosphorylase activity compared to the wild type plants. However, TL46 plants still produced about 40% of the wild type starch levels. If ADPglucose pyrophosphorylase is the rate limiting enzyme, one would have expected a 95% reduction in enzyme activity to produce more than a 60% reduction in starch accumulation. Similarly, the *in vitro* measurements on extractable activities suggest this enzyme can only be rate limiting if its *in vivo* activity is substantially inhibited by the allosteric regulators of the enzyme activity.

SUMMARY OF THE INVENTION

The present invention provides structural DNA constructs which encode an ADPglucose pyrophosphorylase (ADPGPP) enzyme and which are useful in producing enhanced starch content in plants. It is also demonstrated that the ADPGPP enzyme activity in plant cells and tissues is a controlling step in starch biosynthesis.

In accomplishing the foregoing, there is provided, in accordance with one aspect of the present invention, a method of producing genetically transformed plants which have elevated starch content, comprising the steps of:

(a) inserting into the genome of a plant cell a recombinant, double-stranded DNA molecule comprising
  (i) a promoter which functions in plants to cause the production of an RNA sequence in target plant tissues,
  (ii) a structural DNA sequence that causes the production of an RNA sequence which encodes a fusion polypeptide comprising an amino-terminal plastid transit peptide and an ADPglucose pyrophosphorylase enzyme,
  (iii) a 3' non-translated DNA sequence which functions in plant cells to cause transcriptional termination and the addition of polyadenylated nucleotides to the 3' end of the RNA sequence;

(b) obtaining transformed plant cells; and

(c) regenerating from the transformed plant cells genetically transformed plants which have an elevated starch content.

In accordance with another aspect of the present invention, there is provided a recombinant, double-stranded DNA molecule comprising in sequence:

(a) a promoter which functions in plants to cause the production of an RNA sequence in target plant tissues;

(b) a structural DNA sequence that causes the production of an RNA sequence which encodes a fusion polypeptide comprising an amino-terminal plastid transit peptide and an ADPglucose pyrophosphorylase enzyme; and

(c) a 3' non-translated region which functions in plant cells to cause transcriptional termination and the addition of polyadenylated nucleo-tides to the 3' end of the RNA sequence, said promoter being heterologous with respect to the structural DNA.

There has also been provided, in accordance with another aspect of the present invention, bacterial and transformed plant cells that contain, respectively, DNA comprised of the above-mentioned elements (a), (b) and (c).

In accordance with yet another aspect of the present invention, differentiated plants are provided that have increased starch content.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the nucleotide sequence (SEQ ID NO:1) and deduced amino acid sequence (SEQ ID NO:2) for the ADPglucose pyrophosphorylase (*glgC*) gene from *E. coli*.

Figure 2 shows the nucleotide sequence (SEQ ID NO:3) and deduced amino acid sequence (SEQ ID NO:4) for the mutant ADPglucose pyrophosphorylase (*glgC16*) gene from *E. coli*.

Figure 3 shows the nucleotide sequence (SEQ ID NO:5) and corresponding amino acid sequence (SEQ ID NO:6) for the modified chloroplast transit peptide from the ssRUBISCO 1A gene from *Arabidopsis thaliana*.

Figure 4 shows a plasmid map for plant transformation vector pMON530.

Figure 5 shows the nucleotide sequence (SEQ ID NO:7) and the corresponding amino acid sequence (SEQ ID NO:8) of the assembled small subunit ADPglucose pyrophosphorylase gene of potato.

Figure 6 shows the near full length nucleotide sequence (SEQ ID NO:9) and the corresponding amino acid sequence (SEQ ID NO:10) of the almost complete large subunit ADPglucose pyrophosphorylase gene of potato.

Figure 7 shows a plasmid map for plant transformation vector pMON20113.

Figure 8 shows a plasmid map for plant transformation vector pMON16938.

Figure 9 shows a plasmid map for plant transformation vector pMON977.

Figure 10 shows a plasmid map for plant transformation vector pMON16950.

Figure 11 shows a plasmid map for plant transformation vector pMON10098.

DETAILED DESCRIPTION OF THE INVENTION

The expression of a plant gene which exists in double-stranded DNA form involves transcription of messenger RNA (mRNA) from one strand of the DNA by RNA polymerase enzyme, and the subsequent processing of the mRNA primary transcript inside the nucleus. This processing involves a 3' non-translated region which adds polyadenylate nucleotides to the 3' end of the RNA.

Transcription of DNA into mRNA is regulated by a region of DNA usually referred to as the "promoter." The promoter region contains a sequence of bases that signals RNA polymerase to associate with the DNA, and to initiate the transcription of mRNA using one of the DNA strands as a template to make a corresponding complimentary strand of RNA.

A number of promoters which are active in plant cells have been described in the literature. These include the nopaline synthase (NOS) and octopine synthase (OCS) promoters (which are carried on tumor-inducing plasmids of *Agrobacterium tumefaciens*), the caulimovirus promoters such as the cauliflower mosaic virus (CaMV) 19S and 35S and the figwort mosaic virus 35S-promoters, the light-inducible promoter from the small subunit of ribulose-1,5-bis-phosphate carboxylase (ssRUBISCO, a very abundant plant polypeptide), and the chlorophyll a/b binding protein gene promoter, etc. All of these promoters have been used to create various types of DNA

constructs which have been expressed in plants; see, e.g., PCT publication WO 84/02913 (Rogers et al., Monsanto).

Promoters which are known or are found to cause transcription of RNA in plant cells can be used in the present invention. Such promoters may be obtained from a variety of sources such as plants and plant viruses and include, but are not limited to, the enhanced CaMV35S promoter and promoters isolated from plant genes such as ssRUBISCO genes. As described below, it is preferred that the particular promoter selected should be capable of causing sufficient expression to result in the production of an effective amount of ADPglucose pyrophosphorylase enzyme to cause the desired increase in starch content. In addition, it is preferred to bring about expression of the ADPGPP gene in specific tissues of the plant such as leaf, root, tuber, seed, fruit, etc. and the promoter chosen should have the desired tissue and developmental specificity. Those skilled in the art will recognize that the amount of ADPglucose pyrophosphorylase needed to induce the desired increase in starch content may vary with the type of plant and furthermore that too much ADPglucose pyrophosphorylase activity may be deleterious to the plant. Therefore, promoter function should be optimized by selecting a promoter with the desired tissue expression capabilities and approximate promoter strength and selecting a transformant which produces the desired ADPglucose pyrophosphorylase activity in the target tissues. This selection approach from the pool of transformants is routinely employed in expression of heterologous structural genes in plants since there is variation between transformants containing the same heterologous gene due to the site of gene insertion within the plant genome. (Commonly referred to as "position effect").

It is preferred that the promoters utilized in the double-stranded DNA molecules of the present invention have relatively high expression in tissues where the increased starch content is desired, such as the tuber of the potato plant and the fruit of tomato. In potato, a particularly preferred promoter in this regard is the patatin promoter described herein in greater detail in the accompanying examples. Expression of the double-stranded DNA molecules of the present invention by a constitutive promoter, expressing the DNA molecule in all or most of the tissues of the plant, will be rarely preferred and may, in some instances, be detrimental to plant growth.

The class I patatin promoter, used in this study to express the *E. coli* ADPGPP, has been shown to be both highly active and tuber-specific (Bevan et al., 1986; Jefferson et al., 1990). A number of other genes with tuber-specific or enhanced expression are known, including the potato tuber ADPGPP genes (Muller et al., 1990), sucrose synthase (Salanoubat and Belliard, 1987, 1989), the major tuber proteins including the 22 kd protein complexes and proteinase inhibitors (Hannapel, 1990), and the other class I and II patatins (Rocha-Sosa et al., 1989; Mignery et al., 1988).

In addition to the endogenous plant ADPglucose pyrophosphorylase promoters, other promoters can also be used to express an ADPglucose pyrophosphorylase gene in specific tissues, such as leaves, seeds or fruits. ß-conglycinin (also known as the 7S protein) is one of the major storage proteins in soybean (*Glycine max*) (Tierney, 1987). The promoter for ß-conglycinin could be used to over-express the *E. coli*, or any other, ADPglucose pyrophosphorylase gene, specifically in seeds, which would lead to an increase is the starch content of the seeds. The ß-subunit of ß-conglycinin has been expressed,

using its endogenous promoter, in the seeds of transgenic petunia and tobacco, showing that the promoter functions in a seed-specific manner in other plants (Bray, 1987).

The zeins are a group of storage proteins found in maize endosperm. Genomic clones for zein genes have been isolated (Pedersen, 1982), and the promoters from these clones could also be used to express an ADPglucose pyrophosphorylase gene in the seeds of maize and other plants.

The starch content of tomato fruit can be increased by expressing an ADPglucose pyrophosphorylase gene behind a fruit specific promoter. The promoter from the 2A11 genomic clone (Pear, 1989) or the E8 promoter (Deikman, 1988) would express the ADPglucose pyrophosphorylase in tomato fruits. In addition, novel fruit specific promoters exhibiting high and specific expression during the development of the tomato fruit have been isolated. A differential screening approach utilizing a tomato fruit cDNA library was used to identify suitable cDNA clones that expressed specifically in green fruit. cDNA probes prepared from mRNA extracted from fruit at early and late developing stages, from combined leaf+stem tissue, and from root tissue of the tomato plant were used. Clones that expressed abundantly in green fruit and that showed no detectable expression in leaves were identified. Genomic Southern analysis indicated a small (1-2) gene copy number. The promoters for these cDNA clones were then isolated by screening a tomato genomic clone bank. The expression pattern of these promoters is confirmed by fusion to the ß-glucuronidase (GUS) gene and by following the expression of the GUS enzyme during development in transgenic fruit. Promoters that exhibit expression in most cells of the fruit are then fused to the CTP-

glgC16 and other glgC alleles or the ADPGPP genes derived from either algae or plants.

The starch content of root tissue can be increased by expressing an ADPglucose pyrophosphorylase gene behind a root specific promoter. The promoter from the acid chitinase gene (Samac et al., 1990) would express the ADPglucose pyrophosphorylase in root tissue. Expression in root tissue could also be accomplished by utilizing the root specific subdomains of the CaMV35S promoter that have been identified. (Benfey et al., 1989). The starch content of leaf tissue can be increased by expressing the ADPglucose pyrophosphorylase gene (e.g. glgC gene) using a leaf active promoter such as ssRUBISCO promoter or chlorophyll a/b binding protein gene promoter.

The RNA produced by a DNA construct of the present invention also contains a 5' non-translated leader sequence. This sequence can be derived from the promoter selected to express the gene, and can be specifically modified so as to increase translation of the mRNA. The 5' non-translated regions can also be obtained from viral RNAs, from suitable eukaryotic genes, or from a synthetic gene sequence. The present invention is not limited to constructs, as presented in the following examples, wherein the non-translated region is derived from the 5' non-translated sequence that accompanies the promoter sequence. Rather, the non-translated leader sequence can be derived from an unrelated promoter or coding sequence as discussed above.

The DNA constructs of the present invention also contain a structural coding sequence in double-stranded DNA form, which encodes a fusion polypeptide comprising an amino-terminal plastid transit peptide and an ADPglucose pyrophosphorylase enzyme. The ADPglucose pyrophospho-

rylase enzyme utilized in the present invention is preferably subject to reduced allosteric control in plants. Such an unregulated ADPglucose pyrophosphorylase enzyme may be selected from known enzymes which exhibit unregulated enzymatic activity or can be produced by mutagenesis of native bacterial, or algal or plant ADPglucose pyrophosphorylase enzymes as discussed in greater detail hereinafter. In some instances, the substantial differences in the nature of regulators modulating the activity of the wild type ADPglucose pyrophosphorylase (ADPGPP) enzyme permits the use of the wild type gene itself; in these instances, the concentration of the regulators within plant organelles will facilitate elicitation of significant ADPGPP enzyme activity.

Bacterial ADPglucose Pyrophosphorylases

The *E. coli* ADPglucose pyrophosphorylase has been well characterized as a tightly regulated enzyme. The activator fructose 1,6-bisphosphate has been shown to activate the enzyme by increasing its V_{max}, and by increasing the affinity of the enzyme for its substrates (Preiss, 1966 and Gentner, 1967). In addition, fructose 1,6-bisphosphate (FBP) also modulates the sensitivity of the enzyme to the inhibitors adenosine-5'-monophosphate (AMP) and inorganic phosphate (P_i) (Gentner, 1968).

In 1981, the *E. coli* K12 ADPglucose pyrophosphorylase gene (*glg C*), along with the genes for glycogen synthase and branching enzyme, were cloned, and the resulting plasmid was named pOP12 (Okita, 1981). The *glg C* gene, which was sequenced in 1983, contains 1293 bp (SEQ ID NO:1) and encodes 431 amino acids (SEQ ID NO:2) with a deduced molecular weight of 48,762 is shown in Figure 1 (Baecker, 1983).

The *glg C*16 gene was generated by chemically mutagenizing *E. coli* K12 strain PA 601 with N-methyl-N'-nitrosoguanidine (Cattaneo, 1969 and Creuzet-Sigal, 1972). Glycogen biosynthetic mutants were detected by iodine staining of mutagenized colonies. The *glg C*16 mutant was found to accumulate up to 48% glycogen during the stationary phase, compared to 20% glycogen in the parent strain. When the kinetics of the *glg C*16 ADPglucose pyrophosphorylase were compared to the parent, it was found that the *glg C*16 ADPglucose pyrophosphorylase had a higher affinity for ADPglucose in the absence of the activator, Fructose 1,6-bisphosphate (FBP), and the concentration of FBP needed for half-maximal activation of the enzyme was decreased in *glg C*16. The inhibition of the ADPglucose pyrophosphorylase activity in *glg C*16 by 5'-AMP (AMP) was also reduced.

The *glg C*16 gene from *E. coli* K-12 618 has been cloned (Leung, 1986). Two clones, with opposite orientation, were obtained. These clones, pEBL1 and pEBL3, contained both the *glg C*16 and the *glg B* (branching enzyme) genes. Both plasmids were transformed into *E. coli* mutant strains that lacked ADPglucose pyrophosphorylase activity. The *E. coli* K-12 G6MD3 is missing the *glg* genes, while the *E. coli* B strain, AC70R1-504, has a defective ADPglucose pyrophosphorylase gene and is derepressed five- to seven-fold for the other glycogen biosynthetic activities. Both plasmids, pEBL1 and pEBL3, produced ADPglucose pyrophosphorylase activity in both mutant strains. The cloned ADPglucose pyrophosphorylase was partially purified from *E. coli* strain AC70R1 transformed with the pEBL3 plasmid. This enzyme was kinetically compared to partially purified ADPglucose pyrophosphorylase from the original mutant strain (*E. coli* K-12 618), and to the partially purified

ADPglucose pyrophosphorylase from *E. coli* K-12 strain 356, which is the wild type parent strain of strain 618. The wild type and mutant enzymes were compared in their levels of activation and inhibition. The parent strain 356 ADPglucose pyrophosphorylase was activated about 45-fold with fructose 1,6-bisphosphate. The sigmoidal activation curve had a Hill slope of 1.7, and 50% maximal stimulation was seen at 62 μM FBP. The mutant strain 618 ADPglucose pyrophosphorylase was more active in the absence of FBP, and was activated only 1.8- to 2-fold with FBP. The activation curve for the 618 ADPglucose pyrophosphorylase was hyperbolic with a Hill slope of 1.0, and 50% of maximal stimulation was seen at 15 +/-3.1 μM. The enzyme expressed from the pEBL3 plasmid gave the same FBP kinetic constants as the ADPglucose pyrophosphorylase from mutant strain 618.

The DNA sequence of the *glg C*16 gene is now known (SEQ ID NO:3) (Kumar, 1989). Referring to Figure 2, when the *glg C*16 deduced amino acid sequence (SEQ ID NO:4) was compared to the nonisogenic *E. coli* K-12 3000, two amino acid changes are noted. The two changes are Lys 296 to Glu, and Gly 336 to Asp.

A number of other ADPglucose pyrophosphorylase mutants have been found in *E. coli*. The expression of any of these or other bacterial ADPglucose pyrophosphorylase wild type or mutants could also be used to increase starch production in plants.

E. coli K12 strain 6047 (*glg C*47) accumulates about the same amount of glycogen during stationary phase as does strain 618 (*glg C*16). Strain 6047, like 618, shows a higher apparent affinity for FBP, and more activity in the absence of FBP. However, the enzyme from strain 6047 is reportedly more

sensitive to inhibition by AMP compared to the enzyme from strain 618 (Latil-Damotte, 1977).

The *E. coli* B mutant, SG5, has a higher affinity for its allosteric activators and a lower affinity for its allosteric inhibitor, when compared to its parent strain (Govons, 1969; Govons, 1973 and Preiss, 1973). These changes alone make the enzyme more active under physiological conditions, and this causes the bacteria to accumulate two to three times as much glycogen as the parent strain. The mutant ADPglucose pyrophosphorylase from SG5, like the wild type, exists as a homotetramer. Unlike the wild type, however, FBP causes the mutant enzyme to form higher weight oligomers (Carlson, 1976).

The ADPglucose pyrophosphorylase from the *E. coli* B mutant strain CL1136-504 also has a higher apparent affinity for activators and a lower apparent affinity for inhibitors (Kappel, 1981 and Preiss, 1973). This mutant will accumulate three- to four-fold more glycogen than the wild type *E. coli*. Under activated conditions, the purified CL1136-504 enzyme and the wild type (AC70R1) enzyme have comparable specific activities. However, in the absence of any activators, the CL1136-504 enzyme is highly active, unlike the wild type enzyme.

The *glg C* gene from *Salmonella typhimurium* LT2 has also been cloned and sequenced (Leung and Preiss 1987a). The gene encodes 431 amino acids with a deduced molecular weight of 45,580. The *Salmonella typhimurium* LT2 *glg C* gene and the same gene from *E. coli* K-12 have 90% identity at the amino acid level and 80% identity at the DNA level. Like the *E. coli* ADPglucose pyrophosphorylase, the *Salmonella typhimurium* LT2 ADPglucose pyrophosphorylase is also activated by FBP and is inhibited by AMP (Leung and Preiss 1987b). This substantial conservation in amino acid sequences suggests that introduction

of mutations which cause enhancement of ADPGPP activity in *E. coli* into *S. typhimurium* ADPGPP gene should have a similar effect on the ADPGPP enzyme of this organism.

A number of other bacterial ADPglucose pyrophosphorylases have been characterized by their response to activators and inhibitors (for review see: Preiss 1973). Like the *Escherichia coli* ADPglucose pyrophosphorylase, the ADPglucose pyrophosphorylases from *Aerobacter aerogenes*, *Aerobacter cloacae*, *Citrobacter freundii*, and *Escherichia aurescens* are all activated by FBP and are inhibited by AMP. The ADPglucose pyrophosphorylase from *Aeromonas formicans* is activated by fructose 6-phosphate or FBP, and is inhibited by ADP. The *Serratia marcescens* ADPglucose pyrophosphorylase, however, was not activated by any metabolite tested. The photosynthetic *Rhodospirillum rubrum* has an ADPglucose pyrophosphorylase that is activated by pyruvate, and none of the tested compounds, including P_i, AMP or ADP, inhibit the enzyme. Several algal ADPglucose pyrophosphorylases have been studied and found to have regulation similar to that found for plant ADPglucose pyrophosphorylases. Obviously, the ADPglucose pyrophospho-rylases from many organisms could be used to increase starch biosynthesis and accumulation in plants.

In addition to *E. coli* and plant ADPGPP enzymes, other sources, including but not limited to cyanobacteria, algae, and other procaryotic and eucaryotic cells can serve as sources for ADPGPP genes. For example, isolation of the *Synechocystis* and the *Anabaena* ADPGPP genes could be performed using oligonucleotides corresponding to the *E. coli* ADPGPP activator site, (amino acid residues 25-42 of Figure 1), which is highly conserved across widely divergent species. Oligonucleotides

corresponding to this region would facilitate gene isolation when used as probes of genomic libraries. Alternatively, the PCR reaction (described in Example 1) could be used to amplify segments of an ADPGPP gene by using 5' primers corresponding to the *E. coli* activator site, and 3' primers corresponding to *E. coli* catalytic sites, for example, the *E. coli* ADPglucose binding site. Products of the PCR reaction could be used as probes of genomic libraries for isolation of the corresponding full length gene.

Plant ADPglucose Pyrophosphorylases

At one time, UDPglucose was thought to be the primary substrate for starch biosynthesis in plants. However, ADPglucose was found to be a better substrate for starch biosynthesis than UDPglucose (Recondo, 1961). This same report states that ADPglucose pyrophosphorylase activity was found in plant material.

A spinach leaf ADPglucose pyrophosphorylase was partially purified and was shown to be activated by 3-phosphoglycerate (3-PGA) and inhibited by inorganic phosphate (Ghosh et al., 1966). The report by Ghosh et al. suggested that the biosynthesis of leaf starch was regulated by the level of ADPglucose. The activator, 3-PGA, is the primary product of CO_2 fixation in photosynthesis. During photosynthesis, the levels of 3-PGA would increase, causing activation of ADPglucose pyrophosphorylase. At the same time, the levels of P_i would decrease because of photophosphorylation, decreasing the inhibition of ADPglucose pyrophosphorylase. These changes would cause an increase in ADPglucose production and starch biosynthesis. During darkness, 3-PGA levels would decrease,

and P_i levels would increase, decreasing the activity of ADPglucose pyrophosphorylase and, therefore, decreasing biosynthesis of ADPG and starch (Ghosh, 1966).

The ADPglucose pyrophosphorylase from spinach leaves was later purified to homogeneity and shown to contain subunits of 51 and 54 kDa (Morell, 1987). Based on antibodies raised against the two subunits, the 51 kDa protein has homology with both the maize endosperm and potato tuber ADPglucose pyrophosphorylases, but not with the spinach leaf 54 kDa protein.

The sequence of a rice endosperm ADPglucose pyrophosphorylase subunit cDNA clone has been reported (Anderson, 1989a). The clone encoded a protein of 483 amino acids. A comparison of the rice endosperm ADPglucose pyrophosphorylase and the *E. coli* ADPglucose pyrophosphorylase protein sequences shows about 30% identity. Also in 1989, an almost full-length cDNA clone for the wheat endosperm ADPglucose pyrophosphorylase was sequenced (Olive, 1989). The wheat endosperm ADPglucose pyrophosphorylase clone has about 24% identity with the *E. coli* ADPglucose pyrophosphorylase protein sequence, while the wheat and the rice clones have 40% identity at the protein level.

Further evidence for the existence of deregulated wild type plant ADPglucose pyrophosphorylases is found in the paper by Olive et al. (Olive, 1989). They claim that the wheat leaf and endosperm ADPglucose pyrophosphorylases have very different allosteric regulation.. The endosperm ADPglucose pyrophosphorylase is not activated by 3-PGA and requires ten times more of the inhibitor, orthophosphate, to achieve 50% inhibition than the leaf enzyme.

The maize endosperm ADPglucose pyrophosphorylase has been purified and shown to have catalytic and regulatory properties similar to those of other plant ADPglucose pyrophosphorylases (Plaxton, 1987). The native molecular weight of the maize endosperm enzyme is 230,000, and it is composed of four subunits of similar size.

The native molecular weight of the potato tuber ADPglucose pyrophosphorylase is reported to be 200,000, with a subunit size of 50,000 (Sowokinos, 1982). Activity of the tuber ADPglucose pyrophosphorylase is almost completely dependent on 3-PGA, and as with other plant ADPglucose pyrophosphorylases, is inhibited by P_i. The potato tuber and leaf ADPglucose pyrophosphorylases have been demonstrated to be similar in physical, catalytic, and allosteric properties (Anderson, 1989b).

Production of Altered ADPglucose Pyrophosphorylase Genes by Mutagenesis

Those skilled in the art will recognize that while not absolutely required, enhanced results are to be obtained by using ADPglucose pyrophosphorylase genes which are subject to reduced allosteric regulation ("deregulated") and more preferably not subject to significant levels of allosteric regulation ("unregulated") while maintaining adequate catalytic activity. The structural coding sequence for a bacterial or plant ADPglucose pyrophosphorylase enzyme can be mutagenized in *E. coli* or another suitable host and screened for increased glycogen production as described for the *glg C*16 gene of *E. coli*. It should be realized that use of a gene encoding an ADPglucose pyrophosphorylase enzyme which is only subject to modulators (activators/inhibitors) which are present in the selected plant at

levels which do not significantly inhibit the catalytic activity will not require enzyme (gene) modification. These "unregulated" or "deregulated" ADPglucose pyrophosphorylase genes can then be inserted into plants as described herein to obtain transgenic plants having increased starch content.

For example, any ADPglucose pyrophosphorylase gene can be cloned into the *E. coli* B strain AC70R1-504 (Leung, 1986). This strain has a defective ADPglucose pyrophosphorylase gene, and is derepressed five- to seven-fold for the other glycogen biosynthetic enzymes. The ADPglucose pyrophosphorylase gene/cDNA's can be put on a plasmid behind the *E. coli glg C* promoter or any other bacterial promoter. This construct can then be subjected to either site-directed or random mutagenesis. After mutagenesis, the cells would be plated on rich medium with 1% glucose. After the colonies have developed, the plates would be flooded with iodine solution (0.2w/v% I_2, 0.4w/v% KI in H_2O, Creuzet-Sigal, 1972). By comparison with an identical plate containing non-mutated *E. coli*, colonies that are producing more glycogen can be detected by their darker staining.

Since the mutagenesis procedure could have created promoter mutations, any putative ADPglucose pyrophosphorylase mutant from the first round screening will have to have the ADPglucose pyrophosphorylase gene recloned into non-mutated vector and the resulting plasmid will be screened in the same manner. The mutants that make it though both rounds of screening will then have their ADPglucose pyrophosphorylase activities assayed with and without the activators and inhibitors. By comparing the mutated ADPglucose pyrophosphorylase's responses to activators and inhibitors to the non-mutated enzymes, the new mutant can be characterized.

The report by Plaxton and Preiss in 1987 demonstrates that the maize endosperm ADPglucose pyrophosphorylase has regulatory properties similar to those of the other plant ADPglucose pyrophosphorylases (Plaxton and Preiss 1987). They show that earlier reports claiming that the maize endosperm ADPglucose pyrophosphorylase had enhanced activity in the absence of activator (3-PGA) and decreased sensitivity to the inhibitor (P_i), was due to proteolytic cleavage of the enzyme during the isolation procedure. By altering an ADPglucose pyrophosphorylase gene to produce an enzyme analagous to the proteolytically cleaved maize endosperm ADPglucose pyrophosphorylase, decreased allosteric regulation will be achieved.

To assay a liquid culture of *E. coli* for ADPglucose pyrophosphorylase activity, the cells are spun down in a centrifuge and resuspended in about 2 ml of extraction buffer (0.05 M glycylglycine pH 7.0, 5.0 mM DTE, 1.0 mM EDTA) per gram of cell paste. The cells are lysed by passing twice through a French Press. The cell extracts are spun in a microcentrifuge for 5 minutes, and the supernatants are desalted by passing through a G-50 spin column.

The enzyme assay for the synthesis of ADPglucose is a modification of a published procedure (Haugen, 1976). Each 100 µl assay contains: 10 µmole Hepes pH 7.7, 50 µg BSA, 0.05µmole of [^{14}C]glucose-1-phosphate, 0.15 µmole ATP, 0.5 µmole $MgCl_2$, 0.1 µg of crystalline yeast inorganic pyrophosphatase, 1 mM ammonium molybdate, enzyme, activators or inhibitors as desired, and water. The assay is incubated at 37°C for 10 minutes, and is stopped by boiling for 60 seconds. The assay is spun down in a microcentrifuge, and 40 µl of the supernatant is injected onto a Synchrom Synchropak AX-100 anion exchange

HPLC column. The sample is eluted with 65 mM KPi pH 5.5. Unreacted [^{14}C]glucose-1-phosphate elutes around 7-8 minutes, and [^{14}C]ADPglucose elutes at approximately 13 minutes. Enzyme activity is determined by the amount of radioactivity found in the ADPglucose peak.

The plant ADPGPP enzyme activity is tightly regulated, by both positive (3-phosphoglycerate; 3-PGA) and negative effectors (inorganic phosphate; P_i) (Ghosh and Preiss, 1966; Copeland and Preiss 1981; Sowokinos and Preiss 1982; Morell et al., 1987; Plaxton and Preiss, 1987; Preiss, 1988;) and the ratio of 3PGA:P_i plays a prominent role in regulating starch biosynthesis by modulating the ADPGPP activity (Santarius and Heber, 1965; Heldt et al., 1977; Kaiser and Bassham, 1979). The plant ADPGPP enzymes are heterotetramers of two large/"shrunken" and two small/"Brittle" subunits (Morell et al., 1987; Lin et al., 1988a, 1988b; Krishnan et al., 1986; Okita et al., 1990) and there is strong evidence to suggest that the heterotetramer is the most active form of ADPGPP. Support for this suggestion comes from the isolation of plant "starchless" mutants that are deficient in either of the subunits (Tsai and Nelson, 1966; Dickinson and Preiss, 1969; Lin et al., 1988a, 1988b) and from the characterization of an "ADPGPP" homotetramer of small subunits that was found to have only low enzyme activity (Lin et al., 1988b). In addition, proposed effector interaction residues have been identified for both subunits (Morell et al., 1988).

Unregulated enzyme variants of the plant ADPGPP are identified and characterized in a manner similar to that which resulted in the isolation of the *E. coli glgC16* and related mutants. A number of plant ADPGPP cDNA's, or portions of such cDNA's, for both the large and small subunits, have been

cloned from both monocots and dicots (Anderson et al., 1989a; Olive et al., 1989; Muller et al., 1990; Bhave et al., 1990; du Jardin and Berhin, 1991) The proteins encoded by the plant cDNA's, as well as those described from bacteria, show a high degree of conservation (Bhave et al., 1990). In particular, a highly conserved region, also containing some of the residues implicated in enzyme function and effector interactions, has been identified (Morell et al., 1988; du Jardin and Berhin, 1991). Clones of the potato tuber ADPGPP subunit genes have been isolated. These include a complete small subunit gene, assembled by addition of sequences from the first exon of the genomic clone with a nearly full-length cDNA clone of the same gene, and an almost complete gene for the large subunit. The nucleotide sequence (SEQ ID NO:7) and the amino acid sequence (SEQ ID NO:8) of the assembled small subunit gene is presented in Figure 5. The nucleotide sequence presented here differs from the gene originally isolated in the following ways: a *Bgl*II+*Nco*I site was introduced at the ATG codon to facilitate the cloning of the gene into *E. coli* and plant expression vectors by site directed mutagenesis utilizing the oligonucleotide primer sequence

GTTGATAACAAGATCTGTTAACCATGGCGGCTTCC (SEQ ID NO:11).

A *Sac*I site was introduced at the stop codon utilizing the oligonucleotide primer sequence

CCAGTTAAAACGGAGCTCATCAGATGATGATTC (SEQ ID NO:12).

The *Sac*I site serves as a 3' cloning site. An internal *Bgl*II site was removed utilizing the oligonucleotide primer sequence

GTGTGAGAACATAAATCTTGGATATGTTAC (SEQ ID NO:13).

This assembled gene was expressed in *E. coli* under the control of the *rec*A promoter in a P*rec*A-*gene10*L expression cassette (Wong et al., 1988) to produce measurable levels of the protein. An initiating methionine codon is placed by site-directed mutagenesis utilizing the oligonucleotide primer sequence GAATTCACAGGGCCATGGCTCTAGACCC (SEQ ID NO:14) to express the mature gene.

The nucleotide sequence (SEQ ID NO:9) and the amino acid sequence (SEQ ID NO:10) of the almost complete large subunit gene is presented in Figure 6. An initiating methionine codon has been placed at the mature N-terminus by site-directed mutagenesis utilizing the oligonucleotide primer sequence AAGATCAAACCTGCCATGGCTTACTCTGTGATCACTACTG (SEQ ID NO:15). The purpose of the initiating methionine is to facilitate the expression of this large subunit gene in *E. coli*. A *Hind*III site is located 103 bp after the stop codon and serves as the 3' cloning site. The complete large ADPGPP gene is isolated by the 5' RACE procedure (Rapid Amplification of cDNA Ends; Frohman, 1990; Frohman et al., 1988; Loh et al., 1989). The oligonucleotide primers for this procedure are as follows:

1) GGGAATTCAAGCTTGGATCCCGGGCCCCCCCCCCCCCC (SEQ ID NO:16);
2) GGGAATTCAAGCTTGGATCCCGGG (SEQ ID NO:17); and
3) CCTCTAGACAGTCGATCAGGAGCAGATGTACG (SEQ ID NO:18).

The first two are the equivalent to the ANpolyC and the AN primers of Loh et al. (1989), respectively, and the third is the reverse complement to a sequence in the large ADPGPP gene, located after the *Pst* I site in the sequence in Figure 6. The PCR 5' sequence products are cloned as *Eco*RI/*Hind*III/*Bam*HI-*Pst*I

fragments and are easily assembled with the existing gene portion.

The weakly regulated enzyme mutants of ADPGPP are identified by initially scoring colonies from a mutagenized *E. coli* culture that show elevated glycogen synthesis, by iodine staining of 24-48 hour colonies on Luria-Agar plates containing glucose at 1%, and then by characterizing the responses of the ADPGPP enzymes from these isolates to the positive and negative effectors of this activity (Cattaneo et al., 1969; Preiss et al., 1971). A similar approach is applied to the isolation of such variants of the plant ADPGPP enzymes. Given an expression system for each of the subunit genes, mutagenesis of each gene is carried out separately, by any of a variety of known means, both chemical or physical (Miller, 1972) on cultures containing the gene or on purified DNA. Another approach is to use a PCR procedure (Ehrlich, 1989) on the complete gene in the presence of inhibiting Mn^{++} ions, a condition that leads to a high rate of misincorporation of nucleotides. A PCR procedure may also be used with primers adjacent to just a specific region of the gene, and this mutagenized fragment then recloned into the non-mutagenized gene segments. A random synthetic oligonucleotide procedure may also be used to generate a highly mutagenized short region of the gene by mixing of nucleotides in the synthesis reaction to result in misincorporation at all positions in this region. This small region is flanked by restriction sites that are used to reinsert this region into the remainder of the gene. The resultant cultures or transformants are screened by the standard iodine method for those exhibiting glycogen levels higher than controls. Preferably this screening is carried out in an *E. coli* strain deficient only in ADPGPP activity (such as *E. coli* LC618' which is a spontaneous mutant of

LC618 (Cattaneo et al., 1969; Creuzet-Sigal et al., 1972) that is phenotypically glycogen-minus and that is complemented to glycogen-plus by *glgC*. The *E. coli* strain should retain those other activities required for glycogen production. Both genes are expressed together in the same *E. coli* host by placing the genes on compatible plasmids with different selectable marker genes, and these plasmids also have similar copy numbers in the bacterial host to maximize heterotetramer formation. Examples of compatible plasmids include the pBR322/pBR327/pUC series (with Ampicillin selection) based on the ColE1 replicon and the pACYC177 plasmid (with Kanamycin selection) based on the p15A replicon (Chang and Cohen, 1978). The use of separate plasmids enables the screening of a mutagenized population of one gene alone, or in conjunction with the second gene following transformation into a competent host expressing the other gene, and the screening of two mutagenized populations following the combining of these in the same host. Following re-isolation of the plasmid DNA from colonies with increased iodine staining, the ADPGPP coding sequences are recloned into expression vectors, the phenotype verified, and the ADPGPP activity and its response to the effector molecules determined. Improved variants will display increased V_{max}, reduced inhibition by the negative effector (P_i), or reduced dependence upon activator (3-PGA) for maximal activity. The assay for such improved characteristics involves the determination of ADPGPP activity in the presence of P_i at 0.045 mM ($I_{0.5}$ = 0.045 mM) or in the presence of 3-PGA at 0.075 mM ($A_{0.5}$ = 0.075 mM). The useful variants will display <40% inhibition at this concentration of P_i or display >50% activity at this concentration of 3-PGA. Following the isolation of improved variants and the

determination of the subunit or subunits responsible, the mutation(s) are determined by nucleotide sequencing. The mutation is confirmed by recreating this change by site-directed mutagenesis and reassay of ADPGPP activity in the presence of activator and inhibitor. This mutation is then transferred to the equivalent complete ADPGPP cDNA gene, by recloning the region containing the change from the altered bacterial expression form to the plant form containing the amyloplast targeting sequence, or by site-directed mutagenesis of the complete native ADPGPP plant gene.

<u>Chloroplast/Amyloplast Directed Expression of ADPglucose Pyrophos-phorylase Activity</u>

Starch biosynthesis is known to take place in plant chloroplasts and amyloplasts (herein collectively referred to as "plastids". In the plants that have been studied, the ADPglucose pyrophosphorylase is localized to these plastids. ADPglucose pyrophosphorylase is restricted to the chloroplasts in pea shoots (Levi, 1978). In spinach leaves, all of the ADPglucose pyrophosphorylase activity, along with the starch synthase activity, is found in the chloroplasts (Mares, 1978 and Okita, 1979). Immunocytochemical localization shows that the potato tuber ADPglucose pyrophosphorylase is found exclusively in the amyloplasts (Kim, 1989). Studies with rice endosperm also shows that the ADPglucose pyrophosphorylase activity is localized in the amyloplasts (Nakamura, 1989).

Many chloroplast-localized proteins are expressed from nuclear genes as precursors and are targeted to the chloroplast by a chloroplast transit peptide (CTP) that is removed during the import steps. Examples of such chloroplast proteins include the small subunit of Ribulose-1,5-bisphosphate carboxylase

(ssRUBISCO, SSU), 5-enolpyruvateshikimate-3-phosphate synthase (EPSPS), Ferredoxin, Ferredoxin oxidoreductase, the Light-harvesting-complex protein I and protein II, and Thioredoxin F. It has been demonstrated *in vivo* and *in vitro* that non-chloroplast proteins may be targeted to the chloroplast by use of protein fusions with a CTP and that a CTP sequence is sufficient to target a protein to the chloroplast. Likewise, amyloplast-localized proteins are expressed from nuclear genes as precursors and are targeted to the amyloplast by an amyloplast transit peptide (ATP). It is further believed that the chloroplast and amyloplast are developed from common proplastids and are functionally distinct only in that the former is found in photosynthetic cells and the latter in non-photosynthetic cells. In fact, interconversion between the two organella has been observed in plants such as *Picea abies* (Senser, 1975). There are also reports showing that the amyloplast and chloroplast genomes from the same plant are indistinguishable (Scott, 1984; Macherel, 1985 and Catley, 1987). It has been further shown that an amyloplast transit peptide functions to import the associated polypeptide into chloroplasts (Klösgen, 1989).

In the exemplary embodiments, a specialized CTP, derived from the ssRUBISCO 1A gene from *Arabidopsis thaliana* (SSU 1A) (Timko, 1988) was used. This CTP (CTP1) was constructed by a combination of site-directed mutageneses. The CTP1 nucleotide sequence (SEQ ID NO:5) and the corresponding amino acid sequence (SEQ ID NO:6) is also shown in Figure 3. CTP1 is made up of the SSU 1A CTP (amino acid 1-55), the first 23 amino acids of the mature SSU 1A protein (56-78), a serine residue (amino acid 79), a new segment that repeats amino acids 50 to 56 from the CTP and the first two from the mature protein

(amino acids 80-87), and an alanine and methionine residue (amino acid 88 and 89). An *Nco*I restriction site is located at the 3' end (spans the Met codon) to facilitate the construction of precise fusions to the 5' of an ADPglucose pyrophosphorylase gene. At a later stage, a *Bgl*II site was introduced upstream of the N-terminus of the SSU 1A sequences to facilitate the introduction of the fusions into plant transformation vectors. A fusion was assembled between the structural DNA encoding the CTP1 CTP and the *glg C*16 gene from *E. coli* to produce a complete structural DNA sequence encoding the plastid transit peptide/ADPglucose pyrophosphorylase fusion polypeptide.

Those skilled in the art will recognize that if either a single plant ADPglucose pyrophosphorylase cDNA encoding shrunken and/or brittle subunits or both plant ADPGPP cDNA's encoding shrunken and brittle subunits is utilized in the practice of the present invention, the endogenous CTP or ATP could most easily and preferably be used. Hence, for purposes of the present invention the term "plastid transit peptides" should be interpreted to include both chloroplast transit peptides and amyloplast transit peptides. Those skilled in the art will also recognize that various other chimeric constructs can be made which utilize the functionality of a particular plastid transit peptide to import the contiguous ADPglucose pyrophosphorylase enzyme into the plant cell chloroplast/amyloplast depending on the promoter tissue specificity. The functionality of the fusion polypeptide can be confirmed using the following *in vitro* assay.

<u>Plastid Uptake Assay</u>

Intact chloroplasts are isolated from lettuce *(Latuca sativa*, var. longifolia) by centrifugation in Percoll/ficoll gradients as modified from Bartlett et al (1982). The final pellet

of intact chloroplasts is suspended in 0.5 ml of sterile 330 mM sorbitol in 50 mM Hepes-KOH, pH 7.7, assayed for chlorophyll (Arnon, 1949), and adjusted to the final chlorophyll concentration of 4 mg/ml (using sorbitol/Hepes). The yield of intact chloroplasts from a single head of lettuce is 3-6mg chlorophyll.

A typical 300 µl uptake experiment contained 5 mM ATP, 8.3 mM unlabeled methionine, 322 mM sorbitol, 58.3 mM Hepes-KOH (pH 8.0), 50 µl reticulocyte lysate translation products, and intact chloroplasts from *L. sativa* (200 µg chlorophyll). The uptake mixture is gently rocked at room temperature (in 10 x 75 mm glass tubes) directly in front of a fiber optic illuminator set at maximum light intensity (150 Watt bulb). Aliquots of the uptake mix (50 µl) are removed at various times and fractionated over 100 µl silicone-oil gradients (in 150 µl polyethylene tubes) by centrifugation at 11,000 X g for 30 seconds Under these conditions, the intact chloroplasts form a pellet under the silicone-oil layer and the incubation medium (containing the reticulocyte lysate) floats on the surface. After centrifugation, the silicone-oil gradients are immediately frozen in dry ice. The chloroplast pellet is then resuspended in 50-100 µl of lysis buffer (10 mM Hepes-KOH pH 7.5, 1 mM PMSF, 1 mM benzamidine, 5 mM ε-amino-n-caproic acid, and 30 µg/ml aprotinin) and centrifuged at 15,000 X g for 20 minutes to pellet the thylakoid membranes. The clear supernatant (stromal proteins) from this spin, and an aliquot of the reticulocyte lysate incubation medium from each uptake experiment, are mixed with an equal volume of 2X NaDodSO4-PAGE sample buffer for electrophoresis (see below).

SDS-PAGE is carried out according to Laemmli (1970) in 3-17% (w/v) acrylamide slab gels (60 mm X 1.5 mm) with 3%

(w/v) acrylamide stacking gels (5 mm X 1.5 mm). The gel is fixed for 20-30 minutes in a solution with 40% methanol and 10% acetic acid. Then, the gel is soaked in EN³HANCE™ (DuPont)for 20-30 minutes, followed by drying the gel on a gel dryer. The gel is imaged by autoradiography, using an intensifying screen and an overnight exposure to determine whether the ADPglucose pyrophosphorylase is imported into the isolated chloroplasts.

An alternative means for enhancing ADPglucose levels in plant cells will be to isolate genes encoding transcription factors which interact with the upstream regulatory elements of the plant ADPglucose pyrophosphorylase gene(s). Enhanced expression of these transcription factors in plant cells can cause enhanced expression of the ADPglucose pyrophosphorylase gene. Under these conditions, the increased starch content is still realized by an increase in the activity of the ADPglucose pyrophosphorylase enzyme although the mechanism is different. Methods for the isolation of transcription factors have been described (Katagiri, 1989).

Polyadenylation Signal

The 3' non-translated region of the chimeric plant gene contains a polyadenylation signal which functions in plants to cause the addition of polyadenylate nucleotides to the 3' end of the RNA. Examples of suitable 3' regions are (1) the 3' transcribed, non-translated regions containing the polyadenylated signal of *Agrobacterium* the tumor-inducing (Ti) plasmid genes, such as the nopaline synthase (NOS) gene, and (2) plant genes like the soybean storage protein genes and the small subunit of the ribulose-1,5-bisphosphate carboxylase (ssRUBISCO) gene. An example of a preferred 3' region is that

from the NOS gene, described in greater detail in the examples below.

Plant Transformation/Regeneration

Plants which can be made to have increased starch content by practice of the present invention include, but are not limited to, corn, wheat, rice, carrot, onion, pea, tomato, potato, sweet potato, peanut, canola/oilseed rape, barley, sorghum, cassava, banana, soybean, lettuce, apple and walnut.

A double-stranded DNA molecule of the present invention containing the functional plant ADPglucose pyrophosphorylase gene can be inserted into the genome of a plant by any suitable method. Suitable plant transformation vectors include those derived from a Ti plasmid of *Agrobacterium tumefaciens*, as well as those disclosed, e.g., by Herrera-Estrella (1983), Bevan (1983), Klee (1985) and EPO publication 120,516 (Schilperoort et al.). In addition to plant transformation vectors derived from the Ti or root-inducing (Ri) plasmids of *Agrobacterium*, alternative methods can be used to insert the DNA constructs of this invention into plant cells. Such methods may involve, for example, the use of liposomes, electroporation, chemicals that increase free DNA uptake, free DNA delivery via microprojectile bombardment, and transformation using viruses or pollen.

A plasmid expression vector, suitable for the expression of the *E. coli glgC16* and other ADPGPP genes in monocots is composed of the following: a promoter that is specific or enhanced for expression in the starch storage tissues in monocots, generally the endosperm, such as promoters for the zein genes found in the maize endosperm (Pedersen et al., 1982); an intron that provides a splice site to facilitate expression of the

gene, such as the ADH1 intron (Callas et al., 1987); and a 3' polyadenylation sequence such as the nopaline synthase 3' sequence (NOS 3'; Fraley et al., 1983). This expression cassette may be assembled on high copy replicons suitable for the production of large quantities of DNA.

A particularly useful *Agrobacterium*-based plant transformation vector for use in transformation of dicotyledonous plants is plasmid vector pMON530 (Rogers, S.G., 1987). Plasmid pMON530 (see Figure 3) is a derivative of pMON505 prepared by transferring the 2.3 kb StuI-HindIII fragment of pMON316 (Rogers, S.G., 1987) into pMON526. Plasmid pMON526 is a simple derivative of pMON505 in which the SmaI site is removed by digestion with XmaI, treatment with Klenow polymerase and ligation. Plasmid pMON530 retains all the properties of pMON505 and the CaMV35S-NOS expression cassette and now contains a unique cleavage site for SmaI between the promoter and polyadenylation signal.

Binary vector pMON505 is a derivative of pMON200 (Rogers, S.G., 1987) in which the Ti plasmid homology region, LIH, has been replaced with a 3.8 kb HindIII to SmaI segment of the mini RK2 plasmid, pTJS75 (Schmidhauser & Helinski, 1985). This segment contains the RK2 origin of replication, oriV, and the origin of transfer, oriT, for conjugation into *Agrobacterium* using the tri-parental mating procedure (Horsch & Klee, 1986). Plasmid pMON505 retains all the important features of pMON200 including the synthetic multi-linker for insertion of desired DNA fragments, the chimeric NOS/NPTII'/NOS gene for kanamycin resistance in plant cells, the spectinomycin/streptromycin resistance determinant for selection in *E. coli* and *A. tumefaciens*, an intact nopaline synthase gene for facile scoring of transformants and inheritance in progeny and

a pBR322 origin of replication for ease in making large amounts of the vector in *E. coli*. Plasmid pMON505 contains a single T-DNA border derived from the right end of the pTiT37 nopaline-type T-DNA. Southern analyses have shown that plasmid pMON505 and any DNA that it carries are integrated into the plant genome, that is, the entire plasmid is the T-DNA that is inserted into the plant genome. One end of the integrated DNA is located between the right border sequence and the nopaline synthase gene and the other end is between the border sequence and the pBR322 sequences.

When adequate numbers of cells (or protoplasts) containing the ADPglucose pyrophosphorylase gene or cDNA are obtained, the cells (or protoplasts) are regenerated into whole plants. Choice of methodology for the regeneration step is not critical, with suitable protocols being available for hosts from Leguminosae (alfalfa, soybean, clover, etc.), Umbelliferae (carrot, celery, parsnip), Cruciferae (cabbage, radish, rapeseed, etc.), Cucurbitaceae (melons and cucumber), Gramineae (wheat, rice, corn, etc.), Solanaceae (potato, tobacco, tomato, peppers) and various floral crops. See, e.g., Ammirato (1984); Shimamoto, 1989; Fromm, 1990; Vasil, 1990.

The following examples are provided to better elucidate the practice of the present invention and should not be interpreted in any way to limit the scope of the present invention. Those skilled in the art will recognize that various modifications, truncations, etc. can be made to the methods and genes described herein while not departing from the spirit and scope of the present invention.

EXAMPLES

Example 1

To express the *E. coli glg C*16 gene in plant cells, and to target the enzyme to the plastids, the gene needed to be fused to a DNA encoding the plastid-targeting transit peptide (hereinafter referred to as the CTP/ADPglucose pyrophosphorylase gene), and to the proper plant regulatory regions. This was accomplished by cloning the *glg C*16 gene into a series of plasmid vectors that contained the needed sequences.

The plasmid pLP226 contains the *glg C*16 gene on a HincII fragment, cloned into a pUC8 vector at the HincII site (Leung et al. 1986). pLP226 was obtained from Dr. Jack Preiss at Michigan State University, and was transformed into frozen competent *E. coli* JM101 cells, prepared by the calcium chloride method (Sambrook et al., 1989). The transformed cells were plated on 2XYT (infra) plates that contained ampicillin at 100 µg/ml. The plasmid pLP226 was purified by the rapid alkaline extraction procedure (RAE) from a 5 ml overnight culture (Birnboim and Doly 1979).

To fuse the *glg C*16 gene to the DNA encoding the chloroplast transit peptide, a NcoI site was needed at the 5' end of the gene. A SacI site downstream of the termination codon was also needed to move the CTP/ADPglucose pyrophosphorylase gene into the next vector. In order to introduce these sites, a PCR reaction (#13) was run using approximately 20 ng of rapid alkaline extraction-purified plasmid pLP226 for a template. The reaction was set up following the recommendations of the manufacturer (Perkin Elmer Cetus). The primers were QSP3 and QSP7. QSP3 was designed to introduce the NcoI site that would include the start codon for the *glg C* 16 gene. The QSP7 primer hybridized in the

3' nontranslated region of the *glg C*16 gene and added a SacI site. The Thermal Cycler was programmed for 30 cycles with a 1 minute 94°C denaturation step, a 2 minute 50°C annealing step, and a 3 minute 72°C extension step. After each cycle, the extension step was increased by 15 seconds.

QSP3 Primer:
5'-GGAGTTAGCCATGGTTAGTTTAGAG-3' (SEQ ID NO:19)

QSP7 Primer:
5'-GGCCGAGCTCGTCAACGCCGTCTGCGATTTGTGC-3' (SEQ ID NO:20)

The vector that the PCR product was cloned into was pGEM3zf+ (obtained from Promega, Madison, WI) that had been digested with SacI and Hind III, and had the DNA for the modified *Arabidopsis* small subunit CTP1 ligated at the HindIII site. The DNA (SEQ ID NO:5) and amino acid sequence (SEQ ID NO:6) of this CTP1 are shown in Figure 3.

The linearized vector was treated with 5 units of calf intestinal alkaline phosphatase for 30 minutes at 56°C. Then, both the vector and the PCR #13 fragment, which had the *glg C*16 gene with the new NcoI and SacI sites, were run on an agarose gel and the fragments were purified by binding to DEAE membranes. The protocol used for the fragment purification with the DEAE membrane is from Schleicher and Schuell, and is titled "Binding and Recovery of DNA and RNA Using S and S DEAE Membrane."

Ligation #5 fused the *glg C*16 gene to the DNA for the modified *Arabidopsis* SSU CTP with the pGEM3zf+. The ligation contained 3 μl of vector that had been digested with NcoI and

SacI, along with 3 µl of the PCR #13 product, that had also been cut with NcoI and SacI and repurified on a gel. 5 µl (of 20 µl total) of ligation #5 was transformed into frozen competent JM101 cells, and the transformed cells were plated on 2XYT plates (16 g/l Bacto-tryptone, 10 g/l yeast extract, 10 g/l NaCl, pH 7.3, and solidified with 1.5% agar) containing ampicillin.

Sample 1 was picked from a plate after overnight growth. This sample was inoculated into 4 ml of 2XYT media and grown overnight at 37°C. The plasmid was isolated by the rapid alkaline extraction procedure, and the DNA was digested with EcoRI, NcoI, and EcoRI and NcoI together. The digest was separated on an agarose gel, and the expected fragments were observed. The plasmid isolated from sample 1 was designated pMON20100, and consisted of pGEM3zf+, the DNA for the modified *Arabidopsis* SSU CTP, and the *glg C*16 gene. The fusion was in the orientation that allowed it to be transcribed from the SP6 polymerase promoter.

To test this construct for import of the ADPglucose pyrophosphorylase into isolated lettuce chloroplasts, the CTP/ADPglucose pyrophosphorylase fusion needed to be transcribed and translated to produce [^{35}S]-labeled ADPglucose pyrophosphorylase. To make a DNA template for transcription by the SP6 polymerase, the CTP/ADPglucose pyrophosphorylase region of pMON20100 was amplified by PCR to generate a large amount of linear DNA. To do this, about 0.1 µl of pMON20100, that had been purified by rapid alkaline extraction, was used as a template in PCR reaction #80. The primers were a commercially available SP6 promoter primer (Promega) and the oligo QSP7. The SP6 primer hybridized to the SP6 promoter in the vector, and included the entire SP6 promoter sequence. Therefore, a PCR product primed with this oligonucleotide will

contain the recognition sequence for the SP6 polymerase. The QSP7 primer will hybridize in the 3' nontranslated region of the *glg C*16 gene. This is the same primer that was used to introduce a SacI site downstream of the *glg C*16 termination codon. The Thermal Cycler was programmed for 30 cycles with a 1 minute denaturation at 94°C, a 2 minute annealing at 55°C, and a 3 minute extension at 72°C. After each cycle, 15 seconds were added to the extension step.

SP6 Promoter Primer:
5'-GATTTAGGTGACACTATAG-3' (SEQ ID NO:21)

5 µl of PCR reaction #80 was run on an agarose gel and purified by binding to DEAE membrane. The DNA was eluted and dissolved in 20 µl of TE. 2µl of the gel-purified PCR #80 product was used in an SP6 RNA polymerase in vitro transcription reaction. The reaction conditions were those described by the supplier (Promega) for the synthesis of large amounts of RNA (100 µl reaction). The RNA produced from the PCR reaction #80 DNA was used for *in vitro* translation with the rabbit reticulocyte lysate system (Promega). ^{35}S-labeled protein made from pMON20100 (ie:PCR reaction# 80) was used for an in vitro chloroplast import assay as previously described. After processing the samples from the chloroplast import assay, the samples were subjected to electrophoresis on SDS-PAGE gels with a 3-17% polyacrylamide gradient. The gel was fixed for 20-30 minutes in a solution with 40% methanol and 10% acetic acid. Then, the gel was soaked in EN^{3}HANCE™ for 20-30 minutes, followed by drying the gel on a gel dryer. The gel was imaged by autoradiography, using an intensifying screen and

an overnight exposure. The results demonstrated that the fusion protein was imported into the isolated chloroplasts.

The construct in pMON20100 was next engineered to be fused to the En-CaMV35S promoter (Kay, R. 1987) and the NOS 3' end (Bevan, M. 1983) isolated from pMON999. PCR reaction 114 contained plasmid pMON 20100 as a template, and used primers QSM11 and QSM10. QSM11 annealed to the DNA for the modified *Arabidopsis* SSU CTP and created a BglII site 7 bp upstream from the ATG start codon. QSM10 annealed to the 3' end of the *glg C*16 gene and added an XbaI site immediately after the termination codon, and added a SacI site 5 bp after the termination codon. The SacI site that had earlier been added to the *glg C*16 gene was approximately 100 bp downstream of the termination codon. The Thermal Cycler was programmed for 25 cycles with a 1 minute 94°C denaturation, a 2 minute 55°C annealing, and a 3 minute 72°C extension step. With each cycle, 15 seconds was added to the extension step.

QSM11 Primer:
5'-AGAGAGATCTAGAACAATGGCTTCCTCTATGCTCTCTTCCGC-3' (SEQ ID NO:22)

QSM10 Primer:
5'-GGCCGAGCTCTAGATTATCGCTCCTGTTTATGCCCTAAC-3' (SEQ ID NO:23)

Ninety-five (95)µl (from 100 µl total volume) of PCR reaction #114 was ethanol precipitated, and resuspended in 20 µl of TE. Five (5) µl of this was digested with BglII (4 units) and SacI (10 units) overnight at 37°C. Five (5) µl (5 µg) of the vector, pMON999, which contains the En-CaMV35S promoter and the

NOS 3' end, was digested in the same manner. After digestion with the restriction enzymes, the DNAs were run on an agarose gel and purified by binding to DEAE membranes. Each of the DNAs were dissolved in 20 μl of TE. One (1) μl of PCR 114 was ligated with 3 μl of the vector, in a total volume of 20 μl. The ligation mixture was incubated at 14°C for 7 hours. Ten (10) μl of the ligation was transformed into frozen competent MM294 cells and plated on LB plates (10 g/l Bacto-tryptone, 5 g/l yeast extract, 10 g/l NaCl, and 1.5% agar to solidify) with 100 μg/ml ampicillin. Colonies were picked and inoculated into tubes with 5 ml of LB media with 100 μg/ml ampicillin, for overnight growth. The 5 ml overnight cultures were used for rapid alkaline extractions to isolate the plasmid DNAs. The DNAs were digested with EcoRI, and separate aliquots were digested with NotI. After analyzing these samples on agarose gels, the plasmid pMON20102 was confirmed to have the 497 bp EcoRI fragment that is characteristic of the *glg C*16 gene. This plasmid also contained the 2.5 kb NotI fragment which contained the En-CaMV35S promoter, the DNA for the modified *Arabidopsis* SSU CTP, the *glg C*16 gene, and the NOS 3' end.

The 2.5 kb NotI cassette was then transferred into a plant transformation vector, pMON530 (Figure 4). pMON530 contains a unique NotI site in the RK2 region, exactly 600 bp after the HindIII site. A description of the construction of pMON530 can be found in Rogers et al., 1987. Twenty (20) μg of pMON530 was digested with 40 units of NotI overnight at 37°C. The digested vector was then dephosphorylated with 22 units of calf alkaline intestinal phosphatase at 37°C for about 1 hour. The pMON530 vector was extracted with phenol/chloroform, then chloroform, and was ethanol precipitated. Ten (10) μg of plasmid pMON20102 was also digested overnight at 37°C with 40

units of NotI. The NotI-digested pMON530 vector was ligated to the NotI cassette from plasmid pMON20102 at 15°C overnight. The ligation was transformed into frozen competent JM101 *E. coli* cells, and the transformed cells were plated on LB with 75 μg/ml spectinomycin.

Nine colonies were picked from the transformation plate and grown in 5 ml LB cultures for screening. Plasmids from 5 ml cultures were prepared by the rapid alkaline extraction procedure. The DNAs were first screened by SalI digestions which were separated on a 1% agarose gel. By comparing the resulting pattern with the SalI digest of the parent plasmid, pMON530, the correct construct was isolated. The construct was designated pMON20104 and the orientation determined by PstI digestion and NcoI/BglII double digestion. The En-CaMV35S promoter driving the CTP/ADPglucose pyrophosphorylase gene is in the same orientation as the CaMV35S promoter that was already present in pMON530.

In preparation for transforming tobacco cells, pMON20104 was mated into *Agrobacterium* ASE by a triparental mating with the helper plasmid pRK2013. The *Agrobacterium* was grown 1.5 days in LB with 25 μg/ml chloramphenicol and 50 μg/ml kanamycin at 30°C. *E. coli* containing pRK2013 was grown overnight in kanamycin (50 μg/ml). This culture was started with several colonies from a plate. *E. coli* with pMON20104 was grown in LB with 75 μg/ml spectinomycin. After all of the cultures were grown, 4 ml of LB was added to a tube with 100 μl each of *Agrobacterium* ASE, pRK2013, and pMON20104. This mixture was spun in a microfuge for 5 minutes and decanted. The pellet was resuspended in the remaining liquid, and pipetted into the middle of an LB plate. After overnight growth at 30°C, a loop of cells from this plate was

streaked onto an LB plate with 75 μg/ml spectinomycin and 25 μg/ml chloramphenicol.

After 1-2 days at 30°C, the plate from the triparental mating of pMON20104, *Agrobacterium* ASE, and pRK2013, had growing colonies, while the control plate from the mating of pMON20104 and ASE (without pRK2013, which is needed for mobilization) did not. After the triparental mating, 2 colonies were picked from the plate, inoculated into a liquid culture with 75 μg/ml spectinomycin, 25 μg/ml chloramphenicol, and 50 μg/ml kanamycin, and grown at 30°C. These two cultures were used for transformation into tobacco.

The tobacco leaf disc transformation protocol uses healthy leaf tissue about 1 month old. After a 15-20 minute surface sterilization with 10% Clorox plus a surfactant, the leaves were rinsed 3 times in sterile water. Using a sterile paper punch, leaf discs are punched and placed upside down on MS104 media (MS salts 4.3 g/l, sucrose 30 g/l, B5 vitamins 500X 2 ml/l, NAA 0.1 mg/l, and BA 1.0 mg/l) for a 1 day preculture.

The discs were then incolated with an overnight culture of *Agrobacterium* ASE:pMON20104 that had been diluted 1/5 (ie: about 0.6 OD). The inoculation was done by placing the discs in centrifuge tubes with the culture. After 30 to 60 seconds, the liquid was drained off and the discs were blotted between sterile filter paper. The discs were then placed upside down on MS104 feeder plates with a filter disc to co-culture.

After 2-3 days of co-culture, the discs were transferred, still upside down, to selection plates with MS104 media. After 2-3 weeks, callus formed, and individual clumps were separated from the leaf discs. Shoots were cleanly cut from the callus when they were large enough to distinguish from stems. The shoots were placed on hormone-free rooting media (MSO: MS

salts 4.3 g/l, sucrose 30 g/l, and B5 vitamins 500X 2 ml/l) with selection. Roots formed in 1-2 weeks. Rooted shoots were placed in soil and were kept in a high humidity environment (ie: plastic containers or bags). The shoots were hardened off by gradually exposing them to ambient humidity conditions.

Starch levels of transformed callus tissue was quantitated by a modification of the procedure of Lin et al. (Lin et al. 1988a). Clumps of callus were removed from their plates, taking care not to include any agar. The callus was put into 1.5 ml microcentrifuge tubes and dried under a vacuum in a SPEED VAC™ (Savant). After several hours of drying, the tubes were removed and weighed on an analytical balance to the closest 0.1 mg. The tubes were returned to the SPEED VAC™ for several more hours, then were reweighed to determine if a stable dry weight had been obtained. The dried callus was ground in the tube and thoroughly mixed, to give a homogenous sample. An aliquot of each dried callus sample was removed and put into a preweighed 1.5 ml microcentrifuge tube. These new tubes were then reweighed, and the weight of the calli samples in them was determined. The samples ranged from 9 to 34 mg.

Approximately 1 ml of 80% ethanol was added to each tube, and the tubes were incubated in a 70°C water bath for 10-20 minutes. The samples were then spun down, and the ethanol was removed. The ethanol wash was done 2 more times. After the last ethanol wash, the samples were dried in a Speed Vac™, then 200 µl of 0.2 N KOH was added to each tube. The samples were ground using an overhead stirrer, then the samples were heated at 100°C for 30 minutes. Before heating the tubes, several small holes were made in the caps with a needle. This prevented the caps from popping off and causing a loss of sample. After the heating step, 40 µl of 1N acetic acid was added

to each sample. 35 µl (7.4 units) of pancreatic alpha-amylase was added, followed by a 30 minute incubation at 37°C. Next, 5 units (in 5 µl) amyloglucosidase (from *Aspergillus niger*) was added to each sample, along with 160 µl of 100 mM sodium acetate pH. 4.6. The samples were heated to 55°C for 1 hour, boiled for 2-3 minutes, and briefly spun down in a microcentrifuge. At this point, the samples were again dried in a Speed Vac™, and were resuspended in 1000 µl of 100 mM Tris-Cl pH 7.5.

The samples were then assayed for glucose using the Glucose [HK] assay from Sigma (catalogue # 16-10). Using this assay, glucose in the samples (+ATP) is converted to glucose-6-phosphate + ADP by hexokinase. The glucose-6-phosphate (+NAD) is converted to 6-phosphogluconate + NADH. The increase in absorbance at 340 nm, due to NADH, is measured and is directly proportional to the glucose concentration. All assays and calculations were done as recommended by Sigma. The assays were conducted following Sigma's "Alternate Procedure," at room temperature with 10 µl of sample per assay, or 5 µl of sample + 5 µl of 100mM Tris-Cl pH 7.5. The percent starch was determined by dividing the amount (weight) of glucose by the dry weight of the callus.

For the Western blots, a portion of the dried, homogenized callus from each of the 12 samples, plus the 2 control samples, was resuspended in 200 µl of extraction buffer (100 mM Tris-Cl pH 7.1, 1 mM EDTA, 10% glycerol, 5 mM DTT, 1 mM benzamidine). Each sample was ground with an overhead stirrer, spun in a microcentrifuge for 5 minutes at full speed, and the supernatants were removed to new tubes. The protein concentration in each sample was determined by the BioRad protein assay (Lowry et al. 1951), with BSA as a standard.

Twenty-five (25) μg of each sample was loaded onto SDS polyacrylamide gels, with a 7-17% polyacrylamide gradient. Since the samples were loaded onto two gels, the same control callus sample was loaded onto each gel. In addition, a control spiked with 10 ng of pure *E. coli* ADPglucose pyrophosphorylase was loaded onto each gel.

After electrophoresis, the gels were blotted to nitrocellulose using a PolyBlot™ apparatus from American Bionetics. The Western blots were processed according to the protocol provided by Promega. The filters were blocked with 1% BSA in TBST (10 mM Tris-Cl pH 8.0, 150 mM NaCl, and 0.05% Tween 20), for 30 minutes. Ten (10.0) ml of TBST plus 1.3 μl of the primary rabbit anti-*E. coli* ADPglucose pyrophosphorylase antibody were mixed, and the filters was incubated with this primary antibody for 30 minutes. The filters were then washed 3 times with about 50 ml of TBST per wash, for 3 washes of 5 minutes each. Ten (10.0) ml of TBST plus 1.3 μl of the secondary antibody (goat-anti-rabbit conjugated to alkaline phosphatase, Promega) was incubated with the filters for 30 minutes followed again by 3 TBST washes. The signals were visualized using the reaction of alkaline phosphatase with BCIP and NBT, and they were quantitated with a laser densitometer.

Results:

Callus Sample	% Starch	Peak Area
1	26.9%	0.573
2	4.6	0.170
3	6.4	0.0
4	12.3	0.344
5	15.3	0.376
6	11.1	0.314
Control 2 + 10 ng	*	0.369
7	5.5	ND
8	5.6	0.117
9	9.7	0.095
10	6.6	0.0
11	11.4	0.376
12	13.3	0.342
Control 2 + 10 ng	*	0.329
Control 1	3.0	
Control 2	3.7	

* The spiked samples were only used on the Western blots.
ND = not determined

The above results show the results of the quantitative starch assays and the integrated peak areas from the Western blots. The % Starch is reported as the percent of starch relative to the dry weight of the callus. The peak area is the integrated area under the peak from a densitometer scan of the corresponding sample on a Western blot. Samples 1-6 were run on one gel, and samples 7-12 were run on another gel. Control 2

was run on both blots with and without 10 ng of purified *E. coli* ADPglucose pyrophosphorylase. The unspiked samples on both gels showed no interfering bands. The spiked samples had the peak areas shown. These results demonstrate that increased APDglucose leads to increased starch content in plant cells.

Example 2

pMON20104, as described in Example 1, has also been transformed into the Desiree potato strain using the published tuber disc transformation protocol of Sheerman and Bevan (Sheerman and Bevan 1988). Virus-free tubers of *Solanum tuberosum* var. Desiree, were peeled, washed briefly in distilled water, and surface sterilized for 15 minutes in 10% sodium hypochlorite which contained a few drops of Tween 20. The tubers were washed 6 times in sterile water, then were immersed in liquid MS medium. A sterile 1 cm diameter cork borer was used to remove sections of the tubers, and these sections were then cut with a scalpel into 1-2 mm discs. The discs were floated in 20 ml of MS medium containing *Agrobacterium* ASE:pMON20104. A 10 ml culture of *Agrobacterium* ASE:pMON20104 was spun down and resuspended in 20 ml of MS medium before use. The culture and the discs were gently shaken in a petri dish. After 20 minutes, the discs were transferred to tobacco feeder plates with 3C5ZR medium (MS salts, 1 mg/l Thiamine HCl, 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine HCL, 3% sucrose, 5 µM zeatin riboside, and 3 µM IAA aspartic acid, pH 5.9).

After 48 hours, infected discs were put on the new plates with the same medium, but without the feeder layer, and with 500 µg/ml carbenicillin and 100 µg/ml kanamycin. The plates were sealed with parafilm and incubated at 25°C with 16

hours of light/day. The discs were subcultured onto fresh plates every 3 weeks, and the carbenicillin concentration was lowered from 500 to 200 µg/ml after 4 weeks in culture. Developing shoots were removed and placed in large test tubes containing MS salts and R3 vitamins (1 mg/l Thiamine HCl, 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine HCl) plus 200 µg/ml carbenicillin and 100 µg/ml kanamycin. After roots have formed, the plants are transferred to soil and are gradually hardened off.

These preliminary experiments demonstrate that recovering transgenic plants expressing the ADPGPP gene under the control of the En-CaMV35S promoter is problematic. One potato plant was produced on a sucrose containing medium, but when removed from the medium and placed in soil, it did not survive. This result is not unexpected. The En-CaMV35S promoter is a constitutive promoter and causes expression of the ADPGPP in all tissues of the plant. The constitutive expression of the ADPGPP gene most likely causes a deprivation of the sucrose supply to the growing parts of the plant due to the ADPGPP mediated conversion of sucrose to starch in the sugar exporting cells and tissues of the plant. Thus, this example illustrates the expression of ADPGPP in plant cells and the preference, in most cases, that the ADPGPP be expressed specifically in the target tissue, such as the tuber of a potato or the fruit of a tomato. One of ordinary skill in the art would be able to select from a pool of plants transformed with the En-CaMV35S promoter, a plant expressing ADPGPP within the desired range.

<u>Example 3</u>

Potato tissue has also been transformed to express a CTP/ADPglucose pyrophosphorylase fusion polypeptide driven

by a patatin promoter. This construct causes specific expression of the ADPGPP in potato tubers and increases the level of starch in the tubers.

The vector used in the potato transformation is a derivative of the *Agrobacterium* mediated plant transformation vector pMON886. The pMON886 plasmid is made up of the following well characterized segments of DNA. A 0.93 kb fragment isolated from transposon Tn7 which encodes bacterial spectinomycin/streptomycin (Spc/Str) resistance and is a determinant for selection in *E. coli* and *Agrobacterium tumefaciens* (Fling et al., 1985). This is joined to a chimeric kanamycin resistance gene engineered for plant expression to allow selection of the transformed tissue. The chimeric gene consists of the 0.35 kb cauliflower mosaic virus 35S promoter (P-35S) (Odell et al., 1985), the 0.83 kb neomycin phosphotransferase typeII gene (NPTII), and the 0.26 kb 3'-non-translated region of the nopaline synthase gene (NOS 3') (Fraley et al., 1983). The next segment is a 0.75 kb origin of replication from the RK2 plasmid (ori-V) (Stalker et al., 1981). It is joined to a 3.1 kb *Sal*I to *Pvu*I segment of pBR322 which provides the origin of replication for maintenance in *E. coli* (ori-322) and the *bom* site for the conjugational transfer into the *Agrobacterium tumefaciens* cells. Next is a 0.36 kb *Pvu*I fragment from the pTiT37 plasmid which contains the nopaline-type T-DNA right border region (Fraley et al., 1985).

The *glgC16* gene was engineered for expression primarily in the tuber by placing the gene under the control of a tuber-specific promoter. The GlgC16 protein was directed to the plastids within the plant cell due to its synthesis as a C-terminal fusion with a N-terminal protein portion encoding a chloroplast targeting sequence (CTP) derived from that from the SSU 1A

gene from *Arabidopsis thaliana* (Timko et al., 1989). The CTP portion is removed during the import process to liberate the GlgC16 enzyme. Other plant expression signals also include the 3' polyadenylation sequences which are provided by the NOS 3' sequences located downstream from the coding portion of the expression cassette. This cassette was assembled as follows: The patatin promoter was excised from the pBI241.3 plasmid as a *Hind*III-*Bam*HI fragment (The pBI241.3 plasmid contains the patatin-1 promoter segment comprising from the *Acc*I site at 1323 to the *Dra*I site at 2289 [positions refer to the sequence in Bevan et al., 1986] with a *Hind*III linker added at the former and a *Bam*HI linker added at the latter position; Bevan et al., 1986) and ligated together with the CTP1-*glgC16* fusion (the *Bgl*II-*Sac*I fragment from pMON20102 - see Example 1) and pUC-type plasmid vector cut with *Hind*III and *Sac*I (these cloning sites in the vector are flanked by NotI recognition sites). The cassette was then introduced, as a NotI site in pMON886, such that the expression of the *glgC16* gene is in the same orientation as that of the NPTII (kanamycin) gene. This derivative is pMON20113 which is illustrated in Figure 7.

The pMON20113 vector was mobilized into disarmed *Agrobacterium tumefaciens* strain by the triparental conjugation system using the helper plasmid pRK2013 (Ditta et al., 1980). The disarmed strain ABI was used, carrying a Ti plasmid which was disarmed by removing the phytohormone genes responsible for crown gall disease. The ABI strain is the A208 *Agrobacterium tumefaciens* carrying the disarmed pTiC58 plasmid pMP90RK (Koncz and Schell, 1986). The disarmed Ti plasmid provides the trfA gene functions required for autonomous replication of the pMON vector after the conuugation into the ABI strain. When the plant tissue is

incubated with the ABI::pMON conjugate, the vector is transferred to the plant cells by the vir functions encoded by the disarmed pMP90RK Ti plasmid.

The pMON20113 construct is then transformed into the Russet Burbank potato variety. To transform Russet Burbank potatoes, sterile shoot cultures of Russet Burbank are maintained in sundae cups containing 8 ml of PM medium supplemented with 25 mg/L ascorbic acid (Murashige and Skoog (MS) inorganic salts, 30 g/l sucrose, 0.17 g/l $NaH_2PO_4H_2O$, 0.4 mg.l thiamine-HCl, and 100 mg/l myo-inositol, solidified with 2 g/l Gelrite at pH 6.0). When shoots reach approximately 5 cm in length, stem internode segments of 3-5 mm are excised and inoculated with a 1:10 dilution of an overnight culture of *Agrobacterium tumefaciens* from a 4 day old plate culture. The stem explants are co-cultured for 2 days at 20°C on a sterile filter paper placed over 1.5 ml of a tobacco cell feeder layer overlaid on 1/10 P medium (1/10 strength MS inorganic salts and organic addenda without casein as in Jarret et al. (1980), 30 g/l sucrose and 8.0 g/l agar). Following co-culture, the explants are transferred to full strength P-1 medium for callus induction, composed of MS inorganic salts, organic additions as in Jarret et al. (1980), with the exception of casein, 5.0 mg/l zeatin riboside (ZR), and 0.10 mg/l naphthaleneacetic acid NAA (Jarret et al., 1980a, 1980b). Carbenicillin (500 mg/l) and cefotaxime (100 mg/L) are included to inhibit bacterial growth, and 100 mg/l kanamycin is added to select for transformed cells. Transformed potato plants expressing the patatin promoter - CTP/ADPglucose pyrophosphorylase - NOS gene show an increased starch content in the tuber.

After 4 weeks, the explants are transferred to medium of the same composition, but with 0.3 mg/l gibberellic acid (GA3)

replacing the NAA (Jarret et al., 1981) to promote shoot formation. Shoots begin to develop approximately 2 weeks after transfer to shoot induction medium. These shoots are excised and transferred to vials of PM medium for rooting. After about 4 weeks on the rooting medium, the plants are transferred to soil and are gradually hardened off. Shoots are tested for kanamycin resistance conferred by the enzyme neomycin phosphotransferase II, by placing the shoots on PM medium for rooting, which contains 50 mg/L kanamycin, to select for transformed cells.

Russet Burbank Williams plants regenerated in culture were transplanted into 6 inch (~15.24 cm) pots and were grown to maturity under greenhouse condtions. Tubers were harvested and were allowed to suberize at room temperature for two days. All tubers greater than 2 cm. in length were collected and stored at 9°C under high humidity.

Specific gravity (SG) was determined 3 days after harvest for the largest 2 or 3 tubers from each plant, with typical weights being 20-40 grams per tuber. Specific gravity calculations were performed by the weight in air less weight in water method, where SG = weight in air/(weight in air - weight in water). Calculations for percent starch and percent dry matter based on SG were according to the following formulas (von Scheelem, 1937):

$$\% \text{ starch} = 17.546 + (199.07)(SG - 1.0988)$$

$$\% \text{ dry matter} = 24.182 + (211.04)(SG - 1.0988).$$

Western blot analysis was performed on protein extracted from fresh, center sections of tuber tissue as described for tomato leaf tissue. Starch analysis was performed on similar fresh tuber sections as described (Lin, 1988a). Briefly,

approximately 300 mg. center sections were cut, placed in 1.5 ml centrifuge tubes, and frozen on dry ice. The tissue was then dried to a stable weight in a Savant Speed-Vac Concentrator, and final dry weight was determined. Starch content was determined using approximately 60 mg. of dry material from each tuber. Soluble sugars were first removed by extracting three times with 1 ml of 80% ethanol at 70°C, for 20 minutes per treatment. After the final incubation, all remaining ethanol was removed by desiccation in a Speed Vac Concentrator. The solid material was resuspended in 400 µl 0.2 M potassium hydroxide, ground, and then incubated for 30 minutes at 100°C to solubilize the starch. The solutions were cooled and neutralized by addition of 80 µl 1N acetic acid. Starch was degraded to glucose by treatment with 14.8 units of pancreatic alpha-amylase (Sigma Chemical, St. Louis) for 30 minutes at 37°C, followed by 10 units of amyloglucosidase (Sigma Chemical, St. Louis) for 60 minutes at 55°C. Glucose released by the enzymatic digestions was measured using the Sigma Chemical (St. Louis) hexokinase kit.

Western blot and quantitative starch analyses were performed on center cuts from tubers generated under standard greenhouse conditions. Tubers from potato plants expressing *E. coli* ADPGPP contain on average 26.4% higher levels of startch than controls. The range of individual data points shows that two distinct populations exist with respect to starch content. One population, represented by the control tubers, range in starch content from 10.2% up to 15%, with an average starch content of 12.67%. The second population represents expressors of *E. coli* ADPGPP, which range in starch content from 12.1% up to 19.1%, with an average of 16%. The observed increase in starch content correlated with expression levels of *E. coli* ADPGPP,

demonstrating that this expression leads to an increase in starch content in potato tubers.

Specific gravity was determined for the largest 2 or 3 tubers from each of 36 independent transformants by the weight in air less weight in water method (Kleinkopf, 1987). The data show that tubers expressing *E. coli* ADPGPP had a significant increase in specific gravity compared to controls. On average, the specific gravity increased from 1.068 in control tubers up to 1.088 in transgenic tubers (Table 1a), with the best lines averaging specific gravities of about 1.100. Specific gravity values varied among tubers of the same plant, as well as between tubers from different plants, as expected. However, only lines expressing *E. coli* ADPGPP produced tubers with elevated specific gravities, and these increases roughly correlated with the levels of *glgC16* expression. Starch and dry matter content increased on average 35.0% and 23.9% respectively in tubers expressing *E. coli* ADPGPP, with the best lines containing approximately 59.3% and 40.6% increases, respectively.

The starch content determined by the glucose method for a total of 26 potato lines was compared with the starch content calculated for these same tubers using specific gravity measurements. The levels of starch as calculated from specific gravity were in good agreement with that determined directly (Table 1b). For example, tubers expressing *E. coli* ADPGPP contained 16.01% starch as determined by quantitative analysis versus 16.32% as determined by specific gravity. When increases in individual lines were examined, the experimentally determined starch content strongly correlated with the observed increase in dry matter (and expression of the *glgC16* gene). Therefore, the observed increase in dry matter content in tubers

expressing *E. coli* ADPGPP is largely due to the increased deposition of starch.

Table 1

a)	Average Specific Gravity	Average % Starch	Average % Dry Matter
E.coli ADPGPP+ (15)	1.088 (0.012)	15.40	21.90
Controls (21)	1.068 (0.010)	11.41	17.68

The number of plants tested is indicated in parenthesis, with two or three tubers per plant being weighed. Sample standard deviation follows specific gravity (in parenthesis). Percent starch and dry matter were calculated from the average specific gravity as described. Controls consist of a combination of tubers transformed to contain only the DNA vector, without the *glgC16* gene, and tubers from the *glgC16* transformation event which do not express *E. coli* ADPGPP.

b)	Avg % Starch Specific Gravity	Avg % Starch Enzymatic
E.coli ADPGPP+ (11)	16.32 (1.47)	16.01 (2.00)
Controls (15)	11.96 (1.37)	12.67 (1.33)

Average values for percent starch determined experimentally by enzymatic degradation to starch content and calculated from specific gravity measurements. Sample standard deviations are in parenthesis. Differences between *E.coli* ADPGPP+ and controls, calculated by specific gravity or enzymatic methods, are significant at >0.005 level of significance by the Student T-test.

Example 4

The enzyme ADPGPP is encoded by a single gene in *E. coli (glgC)*, whose active form functions as a homotetramer (Preiss, 1984), while the plant enzyme is a heterotetramer encoded by at least two different genes (Copeland and Preiss, 1981). Both *E. coli* and plant ADPGPP's are subject to tight

regulation, with the bacterial enzyme being activated by fructose 1,6-bisphosphate and inhibited by AMP (Preiss, 1984), while the plant enzymes are activated by 3-phosphoglycerate and inhibited by P_i (Copeland and Preiss, 1981; Preiss, 1984). Several mutants of *E. coli* ADPGPP have been characterized and the kinetic properties of a few are summarized and compared in Table 2. (Romeo, T. and Preiss, J., 1989).

Table 2

Strain	Glycogen accumulation (mg/g cells)	Fructose 1,6-biphosphate $A_{0.5}$ (uM)	AMP $I_{0.5}$ (uM)
wild type	20	68	75
SG5	35	22	170
CL1136	74	5.2	680
618	70	15	860

It has been demonstrated that expression of the *glgC16* variant, found in *E. coli* strain 618, leads to enhanced starch biosynthesis in plant cells. Expression of other bacterial ADPGPP enzymes in plant cells also enhance starch content.

Expression of the wild type *glgC* gene also leads to increased starch content. The wild type *glgC* gene, contained on an *E. coli* genomic clone designated pOP12 (Okita et al., 1981) was isolated in a manner similar to that described for the isolation of the *glgC16* gene described in Example 1. Briefly, an *Nco*I site was introduced at the 5' translational start site and a *Sac*I site was introduced just 3' of the termination codon by the PCR reaction using the QSP3 and QSM10 primers described in Example 1. The resultant *Nco*I-*Sac*I fragment was ligated into the vector pMON20102 (described in Example 1) previously

digested with *Nco*I and *Sac*I, giving the plasmid pMON16937. The PS*su-glgC* chimeric gene was constructed by ligating an *Xho*I-*Bgl*II restriction fragment containing the *Ssu1A* promoter (Timko et al., 1985), the *Bgl*II-*Sac*I fragment from pMON16937 comprising the CTP1-*glgC* gene, and the plant transformation vector pMON977 digested with *Xho*I and *Sac*I, to form pMON16938 (Figure 8). The pMON977 plasmid contains the following well characterized DNA segments (Figure 9). First, the 0.93 Kb fragment isolated from transposon Tn7 which encodes bacterial spectinomycin/streptomycin resistance (Spc/Str), and is a determinant for selection in *E. coli* and *Agrobacterium tumefaciens* (Fling et al., 1985). This is joined to the chimeric kanamycin resistance gene engineered for plant expression to allow selection of the transformed tissue. The chimeric gene consists of the 0.35 Kb cauliflower mosaid virus 35S promoter (P-35S)(Odell et al., 1985), the 0.83 Kb neomycin phosphotransferase typeII gene (NPTII), and the 0.26 Kb 3'-nontranslated region of the nopaline synthase gene (NOS 3') (Fraley et al., 1983). The next segment is the 0.75 Kb origin of replication rom the RK2 plasmid (ori-V) (Stalker et al., 1981). This is joined to the 33.1 Kb SalI to PvuI fragment from pBR322 which provides the origin of replication for maintenance in *E. coli* (ori-322), and the *bom* site for the conjugational transfer into the *Agrobacterium tumefaciens* cells. Next is the 0.36 Kb PvuI to BclI fragment from the pTiT37 plasmid, which contains the nopaline-type T-DNA right border region (Fraley et al., 1985). The last segment is the expression cassette consisting of the 0.65 Kb cauliflower mosaic virus (CaMV) 35S promoter enhanced by duplication of the promoter sequence (P-E35S) (Kay et al.,1987), a synthetic multilinker with several unique cloning sites, and the 0.7 Kb 3' nontranslated region of the pea rbcS-E9 gene (E9 3') (Coruzzi et al., 1984; Morelli et al., 1985). The plasmid was

mated into *Agrobacterium tumefaciens* strain ABI, using the triparental mating system, and used to transform *Lycopersicon esculentum* cv. UC82B.

Tomato plant cells are transformed utilizing the *Agrobacterium* strains described above generally by the method as described in McCormick et al. (1986). In particular, cotyledons are obtained from 7-8 day old seedlings. The seeds are surface sterilized for 20 minutes in 30% Clorox bleach and are germinated in Plantcons boxes on Davis germination media. Davis germination media is comprised of 4.3g/l MS salts, 20g/l sucrose and 10 mls/l Nitsch vitamins, pH5.8. The Nitsch vitamin solution is comprised of 100mg/l myo-inositol, 5mg/l nicotinic acid, 0.5mg/l pyridoxine HCl, 0.5mg/l thiamine HCl, 0.05mg/l folic acid, 0.05mg/l biotin, 2mg/l glycine. The seeds are allowed to germinate for 7-8 days in the growth chamber at 25°C, 40% humidity under cool white lights with an intensity of 80 einsteins $m^{-2}s^{-1}$. The photoperiod is 16 hours of light and 8 hours of dark.

Once germination has occurred, the cotyledons are explanted using a #15 feather blade by cutting away the apical meristem and the hypocotyl to create a rectangular explant. These cuts at the short ends of the germinating cotyledon increase the surface area for infection. The explants are bathed in sterile Davis regeneration liquid to prevent desiccation. Davis regeneration media is composed of 1X MS salts, 3% sucrose, 1X Nitsch vitamins, 2.0 mg/l zeatin, pH 5.8. This solution is autoclaved with 0.8% Noble Agar.

The cotyledons are pre-cultured on "feeder plates" composed of Calgene media containing no antibiotics. Calgene media is composed of 4.3g/l MS salts, 30g/l sucrose, 0.1g/l myo-inositol, 0.2g/l KH_2PO_4, 1.45mls/l of a 0.9mg/ml solution of thiamine HCl, 0.2mls of a 0.5mg/ml solution of kinetin and

0.1ml of a 0.2mg/ml solution of 2,4 D, this solution is adjusted to pH 6.0 with KOH. These plates are overlaid with 1.5-2.0 mls of tobacco suspension cells (TXD's) and a sterile Whatman filter which is soaked in 2COO5K media. 2COO5K media is composed of 4.3g/l Gibco MS salt mixture, 1ml B5 vitamins (1000X stock), 30g/l sucrose, 2mls/l PCPA from 2mg/ml stock, and 10µl/l kinetin from 0.5mg/ml stock. The cotyledons are cultured for 1 day in a growth chamber at 25°C under cool white lights with a light intensity of 40-50 einsteins $m^{-2}s^{-1}$ with a continuous light photoperiod.

Cotyledons are then inoculated with a log phase solution of *Agrobacterium* containing the plasmid pMON16938. The concentration of the *Agrobacterium* is approximately $5x10^8$ cells/ml. The cotyledons are allowed to soak in the bacterial solution for six minutes and are then blotted to remove excess solution on sterile Whatman filter disks and are subsequently replaced to the original feeder plate where they are allowed to co-culture for 2 days. After the two days, cotyledons are transferred to selection plates containing Davis regeneration media with 2mg/l zeatin riboside, 500µg/ml carbenicillin, and 100µg/ml kanamycin. After 2-3 weeks, cotyledons with callus and/or shoot formation are transferred to fresh Davis regeneration plates containing carbenicillin and kanamycin at the same levels. The experiment is scored for transformants at this time. The callus tissue is subcultured at regular 3 week intervals and any abnormal structures are trimmed so that the developing shoot buds will continue to regenerate. Shoots develop within 3-4 months.

Once shoots develop, they are excised cleanly from callus tissue and are planted on rooting selection plates. These plates contain 0.5X MSO containing 50µg/ml kanamycin and 500µg/ml carbenicillin. These shoots form roots on the selection

media within two weeks. If no shoots appear after 2 weeks, shoots are trimmed and replanted on the selection media. Shoot cultures are incubated in percivals at a temperature of 22°C. Shoots with roots are then potted when roots are about 2cm in length. The plants are hardened off in a growth chamber at 21°C with a photoperiod of 18 hours light and 6 hours dark for 2-3 weeks prior to transfer to a greenhouse. In the greenhouse, the plants are grown at a temperature of 26°C during the day and 21°C during the night. The photoperiod is 13 hours light and 11 hours dark and allowed to mature.

Transgenic tomato plants transformed with pMON16938 were generated and screened by Western blot analysis for the *glgC* gene product. For Western blot analysis, proteins were extracted from leaf or stem tissue by grinding 1:1 in 100 mM Tris pH7.5, 35 mM KCl, 5 mM dithiothreitol, 5 mM ascorbate, 1 mM EDTA, 1 mM benzamidine, and 20% glycerol. The protein concentration of the extract was determined using the Pierce BCA method, and proteins were separated on 3-17% SDS polyacrylamide gels. *E. coli* ADPGPP was detected using goat antibodies raised against purified *E. coli* ADPGPP and alkaline phosphatase conjugated rabbit anti-goat antibodies (Promega, Madison, WI). In most plants expressing wild type *E. coli* ADPGPP, levels of *E.coli* ADPGPP were on 0.1% of the total extractable protein. For starch analysis, single leaf punches were harvested during late afternoon from 3-4 different, young, fully-expanded leaves per greenhouse grown plant. The leaf punches from each plant were combined and fresh weights were determined using a Mettler analytical balance. Total fresh weight per sample ranged from 60-80 mg. Soluble sugars were first removed by extracting three times with 1 ml of 80% ethanol at 70°C for 20 minutes per treatment. After the final incubation, all remaining ethanol was removed by desiccation in a Speed Vac Concentrator. The solid material was resuspended in 400 µl 0.2 M potassium hydroxide, ground, and then incubated for 30

minutes at 100°C to solubilize the starch. The solutions were cooled and then neutralized by addition of 80 µl 1N acetic acid. Starch was degraded to glucose by treatment with 14.8 units of pancreatic alpha-amylase (Sigma Chemical, St. Louis) for 30 minutes at 37°C, followed by 10 units of amyloglucosidase (Sigma Chemical, St. Louis) for 60 minutes at 55°C. Glucose released by the enzymatic digestions was measured using the Sigma Chemical (St. Louis) hexokinase kit, and these values were used to calculate starch content.

Leaves from tomato plants expressing the *glgC* gene from the Ssu promoter contain on average 29% higher levels of starch than controls, with the best line showing a 107% increase (Table 3).

Table 3

	Average % Starch	Standard Deviation
E. coli ADPGPP+ (7)	4.54	2.1
Controls (8)	3.52	1.9

The number of lines screened are in parentheses.

Thus, other ADPGPP's with different kinetic properties are also effective in increasing starch content in transgenic plants. It should be noted that high level expression of unregulated ADPGPP mutants in leaf tissue is undesiraable since it will cause adverse effects on growth and development of the plants. In fact, use of the *glgC16* gene in place of *glgC* in the above experiments did not result in regeneration of transformants expressing high levels of the *glgC16* gene product.

To express *glgC* from the patatin promoter, the same *Bgl*II-*Sac*I CTP1-*glgC* fragment from pMON16937 and a *Hind*III-*Bam*HI fragment containing the patatin promoter from the

plasmid pBI241.3 were ligated into the binary vector pMON10098 (Figure 11), digested with *Hind*III and *Sac*I, to give the plasmid pMON16950 (Figure 10) The pBI241.3 plasmid contains the patatin-1 promoter segment comprising from the *Acc*I site at 1323 to the *Dra*I site at 22389 [positions refer to the sequence in Bevan et al., 1986] with a *Hind*III linker added at the latter position. The pMON10098 plasmid contains the following DNA regions, moving clockwise around Figure 11. 1) The chimeric kanamycin resistance gene engineered for plant expression to allow selection of the transformed tissue. The chimeric gene consists of the 0.35 Kb cauliflower mosaic virus 35S promoter (P-35S) (Odell et al., 1985), the 0.83 Kb neomycin phosphotransferase typeII gene (KAN), and the 0.26 Kb 3'-nontranslated region of the nopaline synthase gene (NOS 3') (Fraley et al., 1983); 2) The 0.45 Kb ClaI to the DraI fragment from the pTi15955 octopine Ti plasmid, which contains the T-DNA left border region (Barker et al., 1983); 3) The 0.75 Kb segment containing the origin of replication from the RK2 plasmid (ori-V) (Stalker et al., 1981); 4) The 3.0 Kb SalI to PstI segment of pBR322 which provides the origin of replication for maintenance in *E. coli* (ori-322), and the *bom* site for the conjugational transfer into the *Agrobacterium tumefaciens* cells; 5) The 0.93 Kb fragment isolated from transposon Tn7 which encodfes bacterial spectinomycin/streptomycin resistance (Spc/Str) (Fling et al., 1985), and is a determinant for selection in *E. coli* and *Agrobacterium tumefaciens*; 6) The 0.36 Kb PvuI to BclI fragment from the pTiT37 plasmid, which contains the nopaline-type T-DNA right border region (Fraley et al., 1985); and 7) The last segment is the expression cassette consisting of the 0.65 Kb cauliflower mosaic virus (CaMV) 35S promoter enhanced by duplication of the promoter sequence (P-E35S) (Kay et al., 1987), a synthetic multilinker with several unique cloning sites, and the 0.7 Kb 3'

plasmid pBI241.3 were ligated into the binary vector pMON10098 (Figure 11), digested with *Hind*III and *Sac*I, to give the plasmid pMON16950 (Figure 10) The pBI241.3 plasmid contains the patatin-1 promoter segment comprising from the *Acc*I site at 1323 to the *Dra*I site at 22389 [positions refer to the sequence in Bevan et al., 1986] with a *Hind*III linker added at the latter position. The pMON10098 plasmid contains the following DNA regions, moving clockwise around Figure 11. 1) The chimeric kanamycin resistance gene engineered for plant expression to allow selection of the transformed tissue. The chimeric gene consists of the 0.35 Kb cauliflower mosaic virus 35S promoter (P-35S) (Odell et al., 1985), the 0.83 Kb neomycin phosphotransferase typeII gene (KAN), and the 0.26 Kb 3'-nontranslated region of the nopaline synthase gene (NOS 3') (Fraley et al., 1983); 2) The 0.45 Kb ClaI to the DraI fragment from the pTi15955 octopine Ti plasmid, which contains the T-DNA left border region (Barker et al., 1983); 3) The 0.75 Kb segment containing the origin of replication from the RK2 plasmid (ori-V) (Stalker et al., 1981); 4) The 3.0 Kb SalI to PstI segment of pBR322 which provides the origin of replication for maintenance in *E. coli* (ori-322), and the *bom* site for the conjugational transfer into the *Agrobacterium tumefaciens* cells; 5) The 0.93 Kb fragment isolated from transposon Tn7 which encodfes bacterial spectinomycin/streptomycin resistance (Spc/Str) (Fling et al., 1985), and is a determinant for selection in *E. coli* and *Agrobacterium tumefaciens*; 6) The 0.36 Kb PvuI to BclI fragment from the pTiT37 plasmid, which contains the nopaline-type T-DNA right border region (Fraley et al., 1985); and 7) The last segment is the expression cassette consisting of the 0.65 Kb cauliflower mosaic virus (CaMV) 35S promoter enhanced by duplication of the promoter sequence (P-E35S) (Kay et al., 1987), a synthetic multilinker with several unique cloning sites, and the 0.7 Kb 3'

nontranslated region of the pea rbcS-E9 gene (E9 3') (Coruzzi et al., 1984; Morelli et al., 1985). The plasmid was mated into *Agrobacterium tumefaciens* strain ABI, using the triparental mating system, and used to transform Russet Burbank line Williams 82. Expression of *glgC* from the patatin promoter (pMON16950) in potato also results in enhanced starch content in tubers.

In a manner similar to that described for the wild type *glgC* gene and for the *glgC16* mutant gene, the mutant *glgC-SG5* was also expressed in plants and results in an enhancement of starch content.

BIBLIOGRAPHY

Ammirato, P.V., et al. Handbook of Plant Cell Culture - Crop Species. Macmillan Publ. Co. (1984).

Anderson, Joseph M., James Hnilo, Raymond Larson, Thomas W. Okita, Matthew Morell, and Jack Preiss. (1989a) J. Biol. Chem. 264 (21):12238-12242.

Anderson, Joseph M., Thomas W. Okita, Woo Taek Kim, James Hnilo, Joseph Sowokinos, Matthew Morell, and Jack Preiss. (1989b) First International Symposium on the Molecular Biology of the Potato, Bar Harbor, Maine.

Arnon, D.I. (1949) Plant Physiol. 24,1-15

Baecker, Preston A., Clement E. Furlong, and Jack Preiss. (1983) J. Biol. Chem. 258 (8):5084-5087.

Bartlett, S.G., A.R. Grossman, & N.H. Chua. (1982) In Methods in Chloroplast Molecular Biology. Elsevier Biomedical Press, New York, pp 1081-1091.

Beck, Erwin, and Paul Ziegler. (1989) Biosynthesis and Degradation of Starch in Higher Plants. In Annual Review of Plant Physiology and Plant Molecular Biology. 95-117.

Benfey, P., Ren, L., and Chua, N.H. (1989) The EMBO Journal, Vol.5, no.8, pp 2195-2202.

Bevan, M. (1984) Nucleic Acids Res. 12 (22): 8711-8721.

Bevan, M., R. Barker, A. Goldsbrough, M. Jarvis, T. Kavanagh, and G. Iturriaga. (1986) Nucleic Acids Res. 14 (11):4625-4638.

Bhave, M. R., S. Lawrence, C. Barton, and L. C. Hannah. (1990) Plant Cell 2: 581-588.

Birnboim, H.C., and J. Doly. (1979) Nucleic Acids Res. 7 :1513-1523.

Bray, Elizabeth A., Satoshi Naito, Nai-Sui Pan, Edwin Anderson, Philip Dube, and Roger N. Beachy. (1987) Planta 172 364-370.

Callas, J., Fromm, M. and Walbot, V. (1987) Genes and Development 1:1183-1200.

Carlson, Curtis A., Thomas F. Parsons, and Jack Preiss. (1976) J. Biol. Chem. 251 (24):7886-7892.

Catley, M.A., C.W. Bowman, M.W. Bayless and M.D. Gale. (1987) Planta 171:416-421.

Cattaneo, J., M. Damotte, N. Sigal, F. Sanchez-Medina, and J. Puig. (1969) Biochem. Biophys. Res. Commun. 34 (5):694-701.

Chang, A. C. Y. and S.N. Cohen. (1978) J. Bacteriol. 134; 1141-1156.

Copeland, L. and J. Preiss (1981) Plant Physiol. 68: 996-1001.

Creuzet-Sigal, N., M. Latil-Damotte, J. Cattaneo, and J. Puig. (1972) Genetic Analysis and Biochemical Characterization of Mutants Impairing Glycogen Metabolism in *Escherichia coli* K12. In Biochemistry of the Glycosidic Linkage: An Integrated View. Edited by R. Piras and H. G. Pontis. 647-680. New York: Academic Press Inc.

Deikman, J. and R.L. Fischer. (1988) The EMBO Journal 7, 11, 3315-3320.

Dickinson, D. B. and J. Preiss (1969) Plant Physiol. 44:1058-1062.

Ditta, G., Stanfield, S., Corbin, D., and Helinski, D.R. (1980). Broad host range DNA cloning system for Gram-Negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc Natl Acad Sci USA 77, 7347-7351.

Ehrlich, H. A. (1989) Ed. PCR Technology - Principles and Applications for DNA Amplification. Stockton Press, New York.

Fling, M.E., Kopf, J., and Richards, C. (1985). Nucleotide sequence of the transposon Tn7 gene encoding an aminoglycoside-modifying enzyme, 3"(9)-O-nucleotidyltransferase. Nucleic Acids Research 13 no.19, 7095-7106.

Fraley, R.T., Rogers, S.G., Horsch, R.B., Sanders, P.R., Flick, J.S., Adams, S.P., Bittner, M.L., Brand, L.A., Fink, C.L., Fry, J.S., Galluppi, G.R., Goldberg, S.B., Hoffmann, N.L., and Woo, S.C. (1983). Expression of bacterial genes in plant cells. Proc Natl Acad Sci USA 80, 4803-4807.

Fraley, R.T., Rogers, S.G., Horsch, R.B., Eichholtz, D.A., Flick, J.S., Fink, C.L., Hoffmann, N.L., and Sanders, P.R. (1985). The SEV system: a new disarmed Ti plasmid vector system for plant transformation. Bio/Technology 3, 629-635.

Fraley, R., Rogers, S., and Horsch, R. (1986). Genetic transformation in higher plants. Critical Reviews in Plant Sciences 4, No.1, 1-46.

Frohman, M. A., M. K. Rush, and G. R. Martin. (1988) Proc. Natl. Acad. Sci. USA 85: 8998-9002.

Fromm, M., (1990 UCLA Symposium on Molecular Strategies for Crop Improvement, April 16-22, 1990. Keystone, CO.

Gentner, Norman, and Jack Preiss. (1967) Bioch. Biophys. Res. Commun. 27 (3):417-423.

Gentner, Norman, and Jack Preiss. (1968) J. Biol. Chem. 243 (22):5882-5891.

Ghosh, Hara Prasad, and Jack Preiss. (1966) J. Biol. Chem. 241 (19):4491-4504.

Govons, Sydney, Norman Gentner, Elaine Greenberg, and Jack Preiss. (1973) J. Biol. Chem. 248 (5):1731-1740.

Govons, Sydney, Robert Vinopal, John Ingraham, and Jack Preiss. (1969) J. Bact. 97 (2):970-972.

Hannapel, D.J. (1990) Differential expression of potato tuber protein genes. Plant Physiol. 94: 919-925.

Haugen, T.H., A. Ishaque and J. Preiss (1976) J. Biol. Chem. 251. (24) 7880-7885

Heldt, H. W., C. J. Chon, D. Maronde, A. Herold, Z. S. Stankovic, D. Walker, A. Kraminer, M. R. Kirk, and U. Heber. (1977) Plant Physiol. 59: 1146-1155.

Herrera-Estrella, L., et al. (1983) Nature 303:209

Horsch, R.B. and H. Klee. (1986) Proc. Natl. Acad. Sci. U.S.A. 83:4428-32.

Jarret, R. L., Hasegawa, P. M., and Erickson, H. T. (1980a) Physiol. Plant. 49: 177-184.

Jarret, R. L., Hasegawa, P. M., and Erickson, H. T. (1980b) J. Amer. Soc. Hort. Sci. 105: 238-242.

Jarret, R. L., Hasegawa, P. M., and Bressan, R. A. (1981) In Vitro 17: 825-830.

Kaiser, W. M. and J. A. Bassham (1979) Plant Physiol. 63: 109-113.

Kappel, William K., and Jack Preiss. (1981) Arch. Biochem. Biophys. 209 (1):15-28.

Katagiri, F., E. Lam and N. Chua. (1989) Nature 340:727-730.

Kay, R., A. Chan, M. Daly and J. McPherson. (1987) Science 236:1299-1302.

Kim, Woo Taek, Vincent R. Franceschi, Thomas W. Okita, Nina L. Robinson, Matthew Morell, and Jack Preiss. (1989) Plant Physiol. 91 :217-220.

Klee, H.J., et al. (1985) Bio/Technology 3:637-42.

Klee, H.J., and Rogers, S.G. (1989). Plant gene vectors and genetic transformation: plant transformation systems based on the use of *Agrobacterium tumefaciens*. Cell Culture and Somatic Cell, Genetics of Plants 6, 1-23.

Kleinkopf, G.E., Westermann, D.T., Wille, M.J., and Kleinschmidt, G.D. (1987) Specific gravity of Russet burbank potatoes. Am. Potato J. 64: 579-587.

Klösgen, R.B., H. Sandler and J.H. Weil. (1989) Mol. Gen Genet 217:155-166.

Krishnan, H. B., C. D. Reeves, and T. W. Okita (1986) Plant Physiol. 81: 642-645.

Kumar, A., P. Ghosh, M. Young, M. Hill and J. Preiss. (1989) J. Biol. Chem. 264, 18, 10464-10471.

Latil-Damotte, M., and C. Lares. (1977) Mol. Gen. Genet. 150 :325-329.

Lee, Young Moo, Anil Kumar, and Jack Preiss. (1987) Nucleic Acids Res. 15 (24):10603.

Leung, Patrick, Young-Moo Lee, Elaine Greenberg, Keith Esch, Sharon Boylan, and Jack Preiss. (1986) J. Bact. 167 (1):82-88.

Leung, Patrick S. C., and Jack Preiss. (1987a) Biosynthesis of Bacterial Glycogen: Primary Structure of Salmonella typhimurium ADPglucose Synthetase as Deduced from the Nucleotide Sequence of the *glg C* Gene. J. Bact. 169 (9) : 4355-4360.

Leung, Patrick S. C., and Jack Preiss. (1987b). Cloning of the ADPglucose Pyrophosphorylase (*glg C*) and Glycogen Synthase (*glg A*) Structural Genes from Salmonella typhimurium LT2. J. Bact. 169 (9) : 4349-4354.

Levi, Carolyn, and Jack Preiss. (1978) Plant Physiol. 61 :218-220.

Lin, Tsan-Piao, Timothy Caspar, Chris Somerville, and Jack Preiss. (1988a) Plant Physiol. 86 :1131-1135.

Lin, Tsan-Piao, Timothy Caspar, Chris R. Somerville, and Jack Preiss. (1988b) Plant Physiol. 88 :1175-1181.

Loh, E. Y., J. F. Elliott, S. Cwirla, L. L. Lanier, and M. M. Davis. (1989) Science 243:217-220.

Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall. (1951) J. Biol. Chem. 193 :265.

Macherel, D., H. Kobayashi and T. Akazawa. (1985) Biochem. Biophys. Res. Commun. 133:140-146.

Mares, D.J., J.S. Hawker, and J.V. Possingham. (1978) J. of Experimental Botany 29 (111):829-835.

McCormick, S., J. Niedermeyer, J. Fry, A. Barnason, R. Hosrch, and R. Fraley (1986) Plant Cell Reports 5:80-84.

Mignery, Gregory A., Craig S. Pikaard, and William D. Park. (1988) Gene 62:27-44.

Miller, J. H. (1972) Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Morell, M. K., M. Bloom, and J. Preiss. (1988) J. Biol. Chem. 263:633-637.

Morell, Matthew K., Mark Bloom, Vicki Knowles, and Jack Preiss. (1987) Plant Physiol. 85 :182-187.

Muller, B. T., J. Koschmann, L. C. Hannah, L. Willmitzer, and U. Sonnewald (1990) Mol. Gen. Genet. 224:136-146.

Nakamura, Yasunori, Kazuhiro Yuki, Shin-Young Park, and Toshihide Ohya. (1989) Plant Cell Physiol 30 (6): 833-839.

Odell, J.T., Nagy, F., and Chua, N.H. (1985). Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter. Nature 313, 810-812.

Okita, Thomas W., Elaine Greenberg, David N. Kuhn, and Jack Preiss. (1979) Plant Physiol. 64 :187-192.

Okita, Thomas W., Raymond L. Rodriguez, and Jack Preiss. (1981) J. Biol. Chem. 256 (13):6944-6952.

Okita, T. W., P. A. Nakata, J. M. Anderson, J. Sowokinos, M. Morell, and J. Preiss. (1990) 93: 785-790.

Olive, Mark R., R. John Ellis, and Wolfgang Schuch W. (1989) Plant Mol. Biol. 12: 525-538.

Pear, Julie R., Neal Ridge, Rik Rasmussen, Ronald E. Rose, and Catherine M. Houch. (1989) Plant Mol. Biol. 13 :639-651.

Pedersen, Karl, John Devereux, Deborah R. Wilson, Edward Sheldon, and Brian A. Larkins. (1982) Cell 29 : 1015-1026.

Pikaard, Craig S., John S. Brusca, David J. Hannapel, and William D. Park. (1987) Nucleic Acids Res. 15 (5):1979-1994.

Plaxton, William C., and Jack Preiss. (1987) Plant Physiol. 83: 105-112.

Preiss, Jack. (1973) Adenosine Diphosphoryl Glucose Pyrophosphorylase. In Group Transfer. Edited by P. D. Boyer. 73-119. New York: Academic Press.

Preiss, J. (1984) Bacterial glycogen synthesis and its regulation. Annu. Rev. Microbiol. 38: 419-458.

Preiss, Jack. (1988) Biosynthesis of Starch and Its Regulation. In The Biochemistry of Plants. Edited by J. Preiss. 184-249. Orlando, FL: Academic Press.

Preiss, Jack, Laura Shen, Elaine Greenberg, and Norman Gentner. (1966) Biochem. 5 (6):1833-1845.

Preiss, J., A. Sabraw, and E, Greeberg. (1971) Biochem. Biophys. Res. Commun. 42: 180-186.

Recondo, Eduardo, and Luis F. Leloir. (1961) Bioch. Biophys. Res. Commun. 6 (2):85-88.

Rocha-Sosa, Maria, Uwe Sonnewald, Wolf Frommer, Marina Stratmann, Jeff Schell, and Lothar Willmitzer. (1989) EMBO J. 8 (1):23-29.

Rogers, S.G., H.J. Klee, R.B. Horsch, and R.T. Fraley. (1987) Improved Vectors for Plant Transformation: Expression Cassette Vectors and new Selectable Markers. In Methods in Enzymology. Edited by R. Wu and L. Grossman. 253-277. San Diego: Academic Press.

Rogers, S., et al. (1987) In 153 Methods in Enzymology. Edited by H. Weissbach and A. Weissbach. 253: Academic Press.

Rogers, S., and Klee, H. (1987). Pathways to genetic manipulation employing *Agrobacterium*. Plant Gene Research, Plant DNA Infectious Agents, Vol IV. Hohn, T. and J. Schell, eds. Springer-Verlag, Vienna, 179-203.

Romeo, T. and Preiss, J. (1989) Advances in Microbial Physiology, Vol. 30, p. 210.

Rosahl, Sabine, Renate Schmidt, Jeff Schell, and Lothar Willmitzer. (1986) Mol. Gen. Genet. 203 :214-220.

Samac, D.A., C.M. Hironaka, P.E. Yallaly and D.M. Shah (1°90) Plant Physiol. 93:907-914.

Santarius, K. A. and U. Heber (1965) Biochim. Biophys. Acta 102:39-54.

Schmidhauser, T.J. and D.R. Helinski. (1985) J. Bacteriol. 164-155.

Scott, N.S., M.J. Tymms and J.V. Possingham (1984) Planta 161:12-19.

Senser, M., F. Schotz and E. Beck. (1975) Planta 126:1-10.

Sheerman, S., and M.W. Bevan. (1988) Plant Cell Reports 7 :13-16.

Shimamoto, K. et al. (1989) Nature 338:274-276.

Solanoubat, M. and G. Belliard (1987) Molecular cloning and sequencing of sucrose synthase cDNA from potato (*Solanum tuberosum* L.): preliminary characterization of sucrose synthase mRNA distribution. Gene 60:47-56.

Solanoubat, M. and G. Belliard (1989) The steady state level of potato sucrose synthase mRNA is dependent on wounding, anaerobiosis and sucrose concentration. Gene 84:181-185.

Sonnewald, Uwe, Daniel Studer, Mario Rocha-Sosa, and Lothar Willmitzer. (1989) Planta 178 :76-83.

Sowokinos, Joseph R., and Jack Preiss. (1982) Plant Physiol. 69:1459-1466.

Stalker, D.M., Thomas, C.M., and Helinski, D.R. (1981). Nucleotide sequence of the region of the origin of replication of the broad host range plasmid RK2. Mol Gen Genet 181, 8-12.

Tierney, Mary L., Elizabeth Bray A., Randy D. Allen, Yu Ma, Roger F. Drong, Jerry Slightom, and Roger N. Beachy. (1987) Planta 172 :356-363.

Timko, M.P., L. herdies, E. DeAlmeida, A.R. Cashmore, J. Leemans and E. Krebbers. (1988) Genetic Engineering of Nuclear Encoded Components of the Photosynthetic Apparatus in *Arabidopsis*. In Impact of Chemistry on Biotechnology-A Multidisciplinary Discussion. Edited by M. Phillips, S. Shoemaker, R.M. Ottenbrite, R.D. Middlekauff. 279-295. Washington DC: ACS Books.

Timko, M.P., Kausch, A.P., Castresana, C., Fassler, J., Herrera-Estrella, L., Van den Broeck, G., Van Montagu, M., Schell, J., and Cashmore, A.R. (1985) Light regulation of plant gene expression by an upstream enhancer-like element. Nature (London) 318: 579-582.

Tsai, C. Y. and O. E. Nelson. (1966) Science 151: 341-343.

Vasil, V., F. Redway and I. Vasil. (1990) Bio/Technology 8:429-434.

Von Scheele, C. (1937) Die Bestimmung des Starkghelts und der Trockensubstanz der Kartoffel mit hilfe des Specifischen gewichte. Landw Ver Stn 127: 67-96.

Wong, E. Y., Seetharam, R., Kotts, C. E., Heeren, R. A., Klein, B. K., Braford, S. R., Mathis, K. J., Bishop, B. F., Siegel, N. R., Smith, C. E. and Tacon, W. C. (1988) Gene 68: 193-203.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Kishore, Ganesh M.

(ii) TITLE OF INVENTION: Increased Starch Content in Plants

(iii) NUMBER OF SEQUENCES: 23

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Monsanto Co.
(B) STREET: 700 Chesterfield Village Parkway
(C) CITY: St. Louis
(D) STATE: Missouri
(E) COUNTRY: USA
(F) ZIP: 63198

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: McBride, Thomas P.
(B) REGISTRATION NUMBER: 32706
(C) REFERENCE/DOCKET NUMBER: 38-21(10530)A

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (314) 537-7357
(B) TELEFAX: (314) 537-6047

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1296 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1293

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
ATG GTT AGT TTA GAG AAG AAC GAT CAC TTA ATG TTG GCG CGC CAG CTG      48
Met Val Ser Leu Glu Lys Asn Asp His Leu Met Leu Ala Arg Gln Leu
  1             5                  10                  15

CCA TTG AAA TCT GTT GCC CTG ATA CTG GCG GGA GGA CGT GGT ACC CGC      96
```

Pro Leu Lys Ser Val Ala Leu Ile Leu Ala Gly Gly Arg Gly
20 25 30

CTG AAG GAT TTA ACC AAT AAG CGA GCA AAA CCG GCC GTA CAC TTC GGC 144
Leu Lys Asp Leu Thr Asn Lys Arg Ala Lys Pro Ala Val His Phe Gly
35 40 45

GGT AAG TTC CGC ATT ATC GAC TTT GCG CTG TCT AAC TGC ATC AAC TCC 192
Gly Lys Phe Arg Ile Ile Asp Phe Ala Leu Ser Asn Cys Ile Asn Ser
50 55 60

GGG ATC CGT CGT ATG GGC GTG ATC ACC CAG TAC CAG TCC CAC ACT CTG 240
Gly Ile Arg Arg Met Gly Val Ile Thr Gln Tyr Gln Ser His Thr Leu
65 70 75 80

GTG CAG CAC ATT CAG CGC GGC TGG TCA TTC TTC AAT GAA GAA ATG AAC 288
Val Gln His Ile Gln Arg Gly Trp Ser Phe Phe Asn Glu Glu Met Asn
85 90 95

GAG TTT GTC GAT CTG CTG CCA GCA CAG CAG AGA ATG AAA GGG GAA AAC 336
Glu Phe Val Asp Leu Leu Pro Ala Gln Gln Arg Met Lys Gly Glu Asn
100 105 110

TGG TAT CGC GGC ACC GCA GAT GCG GTC ACC CAA AAC CTC GAC ATT ATC 384
Trp Tyr Arg Gly Thr Ala Asp Ala Val Thr Gln Asn Leu Asp Ile Ile
115 120 125

CGT CGT TAT AAA GCG GAA TAC GTG GTG ATC CTG GCG GGC GAC CAT ATC 432
Arg Arg Tyr Lys Ala Glu Tyr Val Val Ile Leu Ala Gly Asp His Ile
130 135 140

TAC AAG CAA GAC TAC TCG CGT ATG CTT ATC GAT CAC GTC GAA AAA GGT 480
Tyr Lys Gln Asp Tyr Ser Arg Met Leu Ile Asp His Val Glu Lys Gly
145 150 155 160

GTA CGT TGT ACC GTT GTT TGT ATG CCA GTA CCG ATT GAA GAA GCC TCC 528
Val Arg Cys Thr Val Val Cys Met Pro Val Pro Ile Glu Glu Ala Ser
165 170 175

GCA TTT GGC GTT ATG GCG GTT GAT GAG AAC GAT AAA ACT ATC GAA TTC 576
Ala Phe Gly Val Met Ala Val Asp Glu Asn Asp Lys Thr Ile Glu Phe
180 185 190

GTG GAA AAA CCT GCT AAC CCG CCG TCA ATG CCG AAC GAT CCG AGC AAA 624
Val Glu Lys Pro Ala Asn Pro Pro Ser Met Pro Asn Asp Pro Ser Lys
195 200 205

TCT CTG GCG AGT ATG GGT ATC TAC GTC TTT GAC GCC GAC TAT CTG TAT 672
Ser Leu Ala Ser Met Gly Ile Tyr Val Phe Asp Ala Asp Tyr Leu Tyr
210 215 220

GAA CTG CTG GAA GAA GAC GAT CGC GAT GAG AAC TCC AGC CAC GAC TTT 720
Glu Leu Leu Glu Glu Asp Asp Arg Asp Glu Asn Ser Ser His Asp Phe
225 230 235 240

GGC AAA GAT TTG ATT CCC AAG ATC ACC GAA GCC GGT CTG GCC TAT GCG 768
Gly Lys Asp Leu Ile Pro Lys Ile Thr Glu Ala Gly Leu Ala Tyr Ala
245 250 255

CAC CCG TTC CCG CTC TCT TGC GTA CAA TCC GAC CCG GAT GCC GAG CCG 816
His Pro Phe Pro Leu Ser Cys Val Gln Ser Asp Pro Asp Ala Glu Pro
260 265 270

TAC TGG CGC GAT GTG GGT ACG CTG GAA GCT TAC TGG AAA GCG AAC CTC 864

```
Tyr Trp Arg Asp Val Gly Thr Leu Glu Ala Tyr Trp Lys Ala A
        275                 280                 285

GAT CTG GCC TCT GTG GTG CCG AAA CTG GAT ATG TAC GAT CGC AAT TGG      912
Asp Leu Ala Ser Val Val Pro Lys Leu Asp Met Tyr Asp Arg Asn Trp
    290                 295                 300

CCA ATT CGC ACC TAC AAT GAA TCA TTA CCG CCA GCG AAA TTC GTG CAG      960
Pro Ile Arg Thr Tyr Asn Glu Ser Leu Pro Pro Ala Lys Phe Val Gln
305                 310                 315                 320

GAT CGC TCC GGT AGC CAC GGG ATG ACC CTT AAC TCA CTG GTT TCC GGC     1008
Asp Arg Ser Gly Ser His Gly Met Thr Leu Asn Ser Leu Val Ser Gly
                325                 330                 335

GGT TGT GTG ATC TCC GGT TCG GTG GTG GTG CAG TCC GTT CTG TTC TCG     1056
Gly Cys Val Ile Ser Gly Ser Val Val Val Gln Ser Val Leu Phe Ser
            340                 345                 350

CGC GTT CGC GTG AAT TCA TTC TGC AAC ATT GAT TCC GCC GTA TTG TTA     1104
Arg Val Arg Val Asn Ser Phe Cys Asn Ile Asp Ser Ala Val Leu Leu
        355                 360                 365

CCG GAA GTA TGG GTA GGT CGC TCG TGC CGT CTG CGC CGC TGC GTC ATC     1152
Pro Glu Val Trp Val Gly Arg Ser Cys Arg Leu Arg Arg Cys Val Ile
    370                 375                 380

GAT CGT GCT TGT GTT ATT CCG GAA GGC ATG GTG ATT GGT GAA AAC GCA     1200
Asp Arg Ala Cys Val Ile Pro Glu Gly Met Val Ile Gly Glu Asn Ala
385                 390                 395                 400

GAG GAA GAT GCA CGT CGT TTC TAT CGT TCA GAA GAA GGC ATC GTG CTG     1248
Glu Glu Asp Ala Arg Arg Phe Tyr Arg Ser Glu Glu Gly Ile Val Leu
                405                 410                 415

GTA ACG CGC GAA ATG CTA CGG AAG TTA GGG CAT AAA CAG GAG CGA         1293
Val Thr Arg Glu Met Leu Arg Lys Leu Gly His Lys Gln Glu Arg
            420                 425                 430

TAA                                                                 1296
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 431 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Val Ser Leu Glu Lys Asn Asp His Leu Met Leu Ala Arg Gln Leu
  1               5                  10                  15

Pro Leu Lys Ser Val Ala Leu Ile Leu Ala Gly Gly Arg Gly Thr Arg
            20                  25                  30

Leu Lys Asp Leu Thr Asn Lys Arg Ala Lys Pro Ala Val His Phe Gly
        35                  40                  45

Gly Lys Phe Arg Ile Ile Asp Phe Ala Leu Ser Asn Cys Ile Asn Ser
    50                  55                  60
```

```
Gly Ile Arg Arg Met Gly Val Ile Thr Gln Tyr Gln Ser His Th
65                  70                  75                  80

Val Gln His Ile Gln Arg Gly Trp Ser Phe Phe Asn Glu Glu Met Asn
                85                  90                  95

Glu Phe Val Asp Leu Leu Pro Ala Gln Gln Arg Met Lys Gly Glu Asn
            100                 105                 110

Trp Tyr Arg Gly Thr Ala Asp Ala Val Thr Gln Asn Leu Asp Ile Ile
        115                 120                 125

Arg Arg Tyr Lys Ala Glu Tyr Val Val Ile Leu Ala Gly Asp His Ile
    130                 135                 140

Tyr Lys Gln Asp Tyr Ser Arg Met Leu Ile Asp His Val Glu Lys Gly
145                 150                 155                 160

Val Arg Cys Thr Val Val Cys Met Pro Val Pro Ile Glu Glu Ala Ser
                165                 170                 175

Ala Phe Gly Val Met Ala Val Asp Glu Asn Asp Lys Thr Ile Glu Phe
            180                 185                 190

Val Glu Lys Pro Ala Asn Pro Pro Ser Met Pro Asn Asp Pro Ser Lys
        195                 200                 205

Ser Leu Ala Ser Met Gly Ile Tyr Val Phe Asp Ala Asp Tyr Leu Tyr
    210                 215                 220

Glu Leu Leu Glu Glu Asp Asp Arg Asp Glu Asn Ser Ser His Asp Phe
225                 230                 235                 240

Gly Lys Asp Leu Ile Pro Lys Ile Thr Glu Ala Gly Leu Ala Tyr Ala
                245                 250                 255

His Pro Phe Pro Leu Ser Cys Val Gln Ser Asp Pro Asp Ala Glu Pro
            260                 265                 270

Tyr Trp Arg Asp Val Gly Thr Leu Glu Ala Tyr Trp Lys Ala Asn Leu
        275                 280                 285

Asp Leu Ala Ser Val Val Pro Lys Leu Asp Met Tyr Asp Arg Asn Trp
    290                 295                 300

Pro Ile Arg Thr Tyr Asn Glu Ser Leu Pro Pro Ala Lys Phe Val Gln
305                 310                 315                 320

Asp Arg Ser Gly Ser His Gly Met Thr Leu Asn Ser Leu Val Ser Gly
                325                 330                 335

Gly Cys Val Ile Ser Gly Ser Val Val Val Gln Ser Val Leu Phe Ser
            340                 345                 350

Arg Val Arg Val Asn Ser Phe Cys Asn Ile Asp Ser Ala Val Leu Leu
        355                 360                 365

Pro Glu Val Trp Val Gly Arg Ser Cys Arg Leu Arg Arg Cys Val Ile
    370                 375                 380

Asp Arg Ala Cys Val Ile Pro Glu Gly Met Val Ile Gly Glu Asn Ala
385                 390                 395                 400
```

Glu Glu Asp Ala Arg Arg Phe Tyr Arg Ser Glu Glu Gly Ile V.
405 410 4?

Val Thr Arg Glu Met Leu Arg Lys Leu Gly His Lys Gln Glu Arg
420 425 430

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1296 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1293

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATG GTT AGT TTA GAG AAG AAC GAT CAC TTA ATG TTG GCG CGC CAG CTG 48
Met Val Ser Leu Glu Lys Asn Asp His Leu Met Leu Ala Arg Gln Leu
1 5 10 15

CCA TTG AAA TCT GTT GCC CTG ATA CTG GCG GGA GGA CGT GGT ACC CGC 96
Pro Leu Lys Ser Val Ala Leu Ile Leu Ala Gly Gly Arg Gly Thr Arg
20 25 30

CTG AAG GAT TTA ACC AAT AAG CGA GCA AAA CCG GCC GTA CAC TTC GGC 144
Leu Lys Asp Leu Thr Asn Lys Arg Ala Lys Pro Ala Val His Phe Gly
35 40 45

GGT AAG TTC CGC ATT ATC GAC TTT GCG CTG TCT AAC TGC ATC AAC TCC 192
Gly Lys Phe Arg Ile Ile Asp Phe Ala Leu Ser Asn Cys Ile Asn Ser
50 55 60

GGG ATC CGT CGT ATG GGC GTG ATC ACC CAG TAC CAG TCC CAC ACT CTG 240
Gly Ile Arg Arg Met Gly Val Ile Thr Gln Tyr Gln Ser His Thr Leu
65 70 75 80

GTG CAG CAC ATT CAG CGC GGC TGG TCA TTC TTC AAT GAA GAA ATG AAC 288
Val Gln His Ile Gln Arg Gly Trp Ser Phe Phe Asn Glu Glu Met Asn
85 90 95

GAG TTT GTC GAT CTG CTG CCA GCA CAG CAG AGA ATG AAA GGG GAA AAC 336
Glu Phe Val Asp Leu Leu Pro Ala Gln Gln Arg Met Lys Gly Glu Asn
100 105 110

TGG TAT CGC GGC ACC GCA GAT GCG GTC ACC CAA AAC CTC GAC ATT ATC 384
Trp Tyr Arg Gly Thr Ala Asp Ala Val Thr Gln Asn Leu Asp Ile Ile
115 120 125

CGT CGT TAT AAA GCG GAA TAC GTG GTG ATC CTG GCG GGC GAC CAT ATC 432
Arg Arg Tyr Lys Ala Glu Tyr Val Val Ile Leu Ala Gly Asp His Ile
130 135 140

TAC AAG CAA GAC TAC TCG CGT ATG CTT ATC GAT CAC GTC GAA AAA GGT 480
Tyr Lys Gln Asp Tyr Ser Arg Met Leu Ile Asp His Val Glu Lys Gly
145 150 155 160

```
GTA CGT TGT ACC GTT GTT TGT ATG CCA GTA CCG ATT GAA GAA G
Val Arg Cys Thr Val Val Cys Met Pro Val Pro Ile Glu Glu A
                165                 170                 175

GCA TTT GGC GTT ATG GCG GTT GAT GAG AAC GAT AAA ACT ATC GAA TTC        576
Ala Phe Gly Val Met Ala Val Asp Glu Asn Asp Lys Thr Ile Glu Phe
            180                 185                 190

GTG GAA AAA CCT GCT AAC CCG CCG TCA ATG CCG AAC GAT CCG AGC AAA        624
Val Glu Lys Pro Ala Asn Pro Pro Ser Met Pro Asn Asp Pro Ser Lys
        195                 200                 205

TCT CTG GCG AGT ATG GGT ATC TAC GTC TTT GAC GCC GAC TAT CTG TAT        672
Ser Leu Ala Ser Met Gly Ile Tyr Val Phe Asp Ala Asp Tyr Leu Tyr
    210                 215                 220

GAA CTG CTG GAA GAA GAC GAT CGC GAT GAG AAC TCC AGC CAC GAC TTT        720
Glu Leu Leu Glu Glu Asp Asp Arg Asp Glu Asn Ser Ser His Asp Phe
225                 230                 235                 240

GGC AAA GAT TTG ATT CCC AAG ATC ACC GAA GCC GGT CTG GCC TAT GCG        768
Gly Lys Asp Leu Ile Pro Lys Ile Thr Glu Ala Gly Leu Ala Tyr Ala
                245                 250                 255

CAC CCG TTC CCG CTC TCT TGC GTA CAA TCC GAC CCG GAT GCC GAG CCG        816
His Pro Phe Pro Leu Ser Cys Val Gln Ser Asp Pro Asp Ala Glu Pro
            260                 265                 270

TAC TGG CGC GAT GTG GGT ACG CTG GAA GCT TAC TGG AAA GCG AAC CTC        864
Tyr Trp Arg Asp Val Gly Thr Leu Glu Ala Tyr Trp Lys Ala Asn Leu
        275                 280                 285

GAT CTG GCC TCT GTG GTG CCG GAA CTG GAT ATG TAC GAT CGC AAT TGG        912
Asp Leu Ala Ser Val Val Pro Glu Leu Asp Met Tyr Asp Arg Asn Trp
    290                 295                 300

CCA ATT CGC ACC TAC AAT GAA TCA TTA CCG CCA GCG AAA TTC GTG CAG        960
Pro Ile Arg Thr Tyr Asn Glu Ser Leu Pro Pro Ala Lys Phe Val Gln
305                 310                 315                 320

GAT CGC TCC GGT AGC CAC GGG ATG ACC CTT AAC TCA CTG GTT TCC GAC       1008
Asp Arg Ser Gly Ser His Gly Met Thr Leu Asn Ser Leu Val Ser Asp
                325                 330                 335

GGT TGT GTG ATC TCC GGT TCG GTG GTG GTG CAG TCC GTT CTG TTC TCG       1056
Gly Cys Val Ile Ser Gly Ser Val Val Val Gln Ser Val Leu Phe Ser
            340                 345                 350

CGC GTT CGC GTG AAT TCA TTC TGC AAC ATT GAT TCC GCC GTA TTG TTA       1104
Arg Val Arg Val Asn Ser Phe Cys Asn Ile Asp Ser Ala Val Leu Leu
        355                 360                 365

CCG GAA GTA TGG GTA GGT CGC TCG TGC CGT CTG CGC CGC TGC GTC ATC       1152
Pro Glu Val Trp Val Gly Arg Ser Cys Arg Leu Arg Arg Cys Val Ile
    370                 375                 380

GAT CGT GCT TGT GTT ATT CCG GAA GGC ATG GTG ATT GGT GAA AAC GCA       1200
Asp Arg Ala Cys Val Ile Pro Glu Gly Met Val Ile Gly Glu Asn Ala
385                 390                 395                 400

GAG GAA GAT GCA CGT CGT TTC TAT CGT TCA GAA GAA GGC ATC GTG CTG       1248
Glu Glu Asp Ala Arg Arg Phe Tyr Arg Ser Glu Glu Gly Ile Val Leu
                405                 410                 415
```

GTA ACG CGC GAA ATG CTA CGG AAG TTA GGG CAT AAA CAG GAG
Val Thr Arg Glu Met Leu Arg Lys Leu Gly His Lys Gln Glu
420 425 430

TAA 1296

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 431 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Val Ser Leu Glu Lys Asn Asp His Leu Met Leu Ala Arg Gln Leu
1 5 10 15

Pro Leu Lys Ser Val Ala Leu Ile Leu Ala Gly Gly Arg Gly Thr Arg
20 25 30

Leu Lys Asp Leu Thr Asn Lys Arg Ala Lys Pro Ala Val His Phe Gly
35 40 45

Gly Lys Phe Arg Ile Ile Asp Phe Ala Leu Ser Asn Cys Ile Asn Ser
50 55 60

Gly Ile Arg Arg Met Gly Val Ile Thr Gln Tyr Gln Ser His Thr Leu
65 70 75 80

Val Gln His Ile Gln Arg Gly Trp Ser Phe Phe Asn Glu Glu Met Asn
85 90 95

Glu Phe Val Asp Leu Leu Pro Ala Gln Gln Arg Met Lys Gly Glu Asn
100 105 110

Trp Tyr Arg Gly Thr Ala Asp Ala Val Thr Gln Asn Leu Asp Ile Ile
115 120 125

Arg Arg Tyr Lys Ala Glu Tyr Val Val Ile Leu Ala Gly Asp His Ile
130 135 140

Tyr Lys Gln Asp Tyr Ser Arg Met Leu Ile Asp His Val Glu Lys Gly
145 150 155 160

Val Arg Cys Thr Val Val Cys Met Pro Val Pro Ile Glu Glu Ala Ser
165 170 175

Ala Phe Gly Val Met Ala Val Asp Glu Asn Asp Lys Thr Ile Glu Phe
180 185 190

Val Glu Lys Pro Ala Asn Pro Pro Ser Met Pro Asn Asp Pro Ser Lys
195 200 205

Ser Leu Ala Ser Met Gly Ile Tyr Val Phe Asp Ala Asp Tyr Leu Tyr
210 215 220

Glu Leu Leu Glu Glu Asp Asp Arg Asp Glu Asn Ser Ser His Asp Phe
225 230 235 240

Gly Lys Asp Leu Ile Pro Lys Ile Thr Glu Ala Gly Leu Ala Tyr Ala

```
                245                     250

His Pro Phe Pro Leu Ser Cys Val Gln Ser Asp Pro Asp Ala Glu Pro
            260                 265                 270

Tyr Trp Arg Asp Val Gly Thr Leu Glu Ala Tyr Trp Lys Ala Asn Leu
        275                 280                 285

Asp Leu Ala Ser Val Val Pro Glu Leu Asp Met Tyr Asp Arg Asn Trp
    290                 295                 300

Pro Ile Arg Thr Tyr Asn Glu Ser Leu Pro Pro Ala Lys Phe Val Gln
305                 310                 315                     320

Asp Arg Ser Gly Ser His Gly Met Thr Leu Asn Ser Leu Val Ser Asp
                325                 330                 335

Gly Cys Val Ile Ser Gly Ser Val Val Val Gln Ser Val Leu Phe Ser
            340                 345                 350

Arg Val Arg Val Asn Ser Phe Cys Asn Ile Asp Ser Ala Val Leu Leu
        355                 360                 365

Pro Glu Val Trp Val Gly Arg Ser Cys Arg Leu Arg Arg Cys Val Ile
    370                 375                 380

Asp Arg Ala Cys Val Ile Pro Glu Gly Met Val Ile Gly Glu Asn Ala
385                 390                 395                     400

Glu Glu Asp Ala Arg Arg Phe Tyr Arg Ser Glu Glu Gly Ile Val Leu
                405                 410                 415

Val Thr Arg Glu Met Leu Arg Lys Leu Gly His Lys Gln Glu Arg
            420                 425                 430
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 355 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 88..354

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
AAGCTTGTTC TCATTGTTGT TATCATTATA TATAGATGAC CAAAGCACTA GACCAAACCT        60

CAGTCACACA AAGAGTAAAG AAGAACA ATG GCT TCC TCT ATG CTC TCT TCC           111
                              Met Ala Ser Ser Met Leu Ser Ser
                               1               5

GCT ACT ATG GTT GCC TCT CCG GCT CAG GCC ACT ATG GTC GCT CCT TTC         159
Ala Thr Met Val Ala Ser Pro Ala Gln Ala Thr Met Val Ala Pro Phe
     10                  15                  20

AAC GGA CTT AAG TCC TCC GCT GCC TTC CCA GCC ACC CGC AAG GCT AAC         207
```

```
Asn Gly Leu Lys Ser Ser Ala Ala Phe Pro Ala Thr Arg Lys
 25                 30                  35

AAC GAC ATT ACT TCC ATC ACA AGC AAC GGC GGA AGA GTT AAC TGC ATG      255
Asn Asp Ile Thr Ser Ile Thr Ser Asn Gly Gly Arg Val Asn Cys Met
                 45                  50                  55

CAG GTG TGG CCT CCG ATT GGA AAG AAG AAG TTT GAG ACT CTC TCT TAC      303
Gln Val Trp Pro Pro Ile Gly Lys Lys Lys Phe Glu Thr Leu Ser Tyr
             60                  65                  70

CTT CCT GAC CTT ACC GAT TCC GGT GGT CGC GTC AAC TGC ATG CAG GCC      351
Leu Pro Asp Leu Thr Asp Ser Gly Gly Arg Val Asn Cys Met Gln Ala
         75                  80                  85

ATG G                                                                355
Met
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 89 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Met Ala Ser Ser Met Leu Ser Ser Ala Thr Met Val Ala Ser Pro Ala
 1               5                  10                  15

Gln Ala Thr Met Val Ala Pro Phe Asn Gly Leu Lys Ser Ser Ala Ala
             20                  25                  30

Phe Pro Ala Thr Arg Lys Ala Asn Asn Asp Ile Thr Ser Ile Thr Ser
         35                  40                  45

Asn Gly Gly Arg Val Asn Cys Met Gln Val Trp Pro Pro Ile Gly Lys
     50                  55                  60

Lys Lys Phe Glu Thr Leu Ser Tyr Leu Pro Asp Leu Thr Asp Ser Gly
 65                  70                  75                  80

Gly Arg Val Asn Cys Met Gln Ala Met
                 85
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1575 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 3..1565

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
CC ATG GCG GCT TCC ATT GGA GCC TTA AAA TCT TCA CCT TCT TCT AAC      47
   Met Ala Ala Ser Ile Gly Ala Leu Lys Ser Ser Pro Ser Ser Asn
    1               5                  10                  15

AAT TGC ATC AAT GAG AGA AGA AAT GAT TCT ACA CGT GCT GTA TCC AGC     95
Asn Cys Ile Asn Glu Arg Arg Asn Asp Ser Thr Arg Ala Val Ser Ser
                20                  25                  30

AGA AAT CTC TCA TTT TCG TCT TCT CAT CTC GCC GGA GAC AAG TTG ATG    143
Arg Asn Leu Ser Phe Ser Ser Ser His Leu Ala Gly Asp Lys Leu Met
            35                  40                  45

CCT GTA TCG TCC TTA CGT TCC CAA GGA GTC CGA TTC AAT GTG AGA AGA    191
Pro Val Ser Ser Leu Arg Ser Gln Gly Val Arg Phe Asn Val Arg Arg
        50                  55                  60

AGT CCA ATG ATT GTG TCG CCA AAG GCT GTT TCT GAT TCG CAG AAT TCA    239
Ser Pro Met Ile Val Ser Pro Lys Ala Val Ser Asp Ser Gln Asn Ser
    65                  70                  75

CAG ACA TGT CTA GAC CCA GAT GCT AGC CGG AGT GTT TTG GGA ATT ATT    287
Gln Thr Cys Leu Asp Pro Asp Ala Ser Arg Ser Val Leu Gly Ile Ile
 80                  85                  90                  95

CTT GGA GGT GGA GCT GGG ACC CGA CTT TAT CCT CTA ACT AAA AAA AGA    335
Leu Gly Gly Gly Ala Gly Thr Arg Leu Tyr Pro Leu Thr Lys Lys Arg
                100                 105                 110

GCA AAG CCA GCT GTT CCA CTT GGA GCA AAT TAT CGT CTG ATT GAC ATT    383
Ala Lys Pro Ala Val Pro Leu Gly Ala Asn Tyr Arg Leu Ile Asp Ile
            115                 120                 125

CCT GTA AGC AAC TGC TTG AAC AGT AAT ATA TCC AAG ATT TAT GTT CTC    431
Pro Val Ser Asn Cys Leu Asn Ser Asn Ile Ser Lys Ile Tyr Val Leu
        130                 135                 140

ACA CAA TTC AAC TCT GCC TCT CTG AAT CGC CAC CTT TCA CGA GCA TAT    479
Thr Gln Phe Asn Ser Ala Ser Leu Asn Arg His Leu Ser Arg Ala Tyr
    145                 150                 155

GCT AGC AAC ATG GGA GGA TAC AAA AAC GAG GGC TTT GTG GAA GTT CTT    527
Ala Ser Asn Met Gly Gly Tyr Lys Asn Glu Gly Phe Val Glu Val Leu
160                 165                 170                 175

GCT GCT CAA CAA AGT CCA GAG AAC CCC GAT TGG TTC CAG GGC ACG GCT    575
Ala Ala Gln Gln Ser Pro Glu Asn Pro Asp Trp Phe Gln Gly Thr Ala
                180                 185                 190

GAT GCT GTC AGA CAA TAT CTG TGG TTG TTT GAG GAG CAT ACT GTT CTT    623
Asp Ala Val Arg Gln Tyr Leu Trp Leu Phe Glu Glu His Thr Val Leu
            195                 200                 205

GAA TAC CTT ATA CTT GCT GGA GAT CAT CTG TAT CGA ATG GAT TAT GAA    671
Glu Tyr Leu Ile Leu Ala Gly Asp His Leu Tyr Arg Met Asp Tyr Glu
        210                 215                 220

AAG TTT ATT CAA GCC CAC AGA GAA ACA GAT GCT GAT ATT ACC GTT GCC    719
Lys Phe Ile Gln Ala His Arg Glu Thr Asp Ala Asp Ile Thr Val Ala
    225                 230                 235

GCA CTG CCA ATG GAC GAG AAG CGT GCC ACT GCA TTC GGT CTC ATG AAG    767
```

```
Ala Leu Pro Met Asp Glu Lys Arg Ala Thr Ala Phe Gly Leu
240                 245                 250

ATT GAC GAA GAA GGA CGC ATT ATT GAA TTT GCA GAG AAA CCG CAA GGA      815
Ile Asp Glu Glu Gly Arg Ile Ile Glu Phe Ala Glu Lys Pro Gln Gly
                260                 265                 270

GAG CAA TTG CAA GCA ATG AAA GTG GAT ACT ACC ATT TTA GGT CTT GAT      863
Glu Gln Leu Gln Ala Met Lys Val Asp Thr Thr Ile Leu Gly Leu Asp
            275                 280                 285

GAC AAG AGA GCT AAA GAA ATG CCT TTC ATT GCC AGT ATG GGT ATA TAT      911
Asp Lys Arg Ala Lys Glu Met Pro Phe Ile Ala Ser Met Gly Ile Tyr
        290                 295                 300

GTC ATT AGC AAA GAC GTG ATG TTA AAC CTA CTT CGT GAC AAG TTC CCT      959
Val Ile Ser Lys Asp Val Met Leu Asn Leu Leu Arg Asp Lys Phe Pro
    305                 310                 315

GGG GCC AAT GAT TTT GGT AGT GAA GTT ATT CCT GGT GCA ACT TCA CTT     1007
Gly Ala Asn Asp Phe Gly Ser Glu Val Ile Pro Gly Ala Thr Ser Leu
320                 325                 330                 335

GGG ATG AGA GTG CAA GCT TAT TTA TAT GAT GGG TAC TGG GAA GAT ATT     1055
Gly Met Arg Val Gln Ala Tyr Leu Tyr Asp Gly Tyr Trp Glu Asp Ile
                340                 345                 350

GGT ACC ATT GAA GCT TTC TAC AAT GCC AAT TTG GGC ATT ACA AAA AAG     1103
Gly Thr Ile Glu Ala Phe Tyr Asn Ala Asn Leu Gly Ile Thr Lys Lys
            355                 360                 365

CCG GTG CCA GAT TTT AGC TTT TAC GAC CGA TCA GCC CCA ATC TAC ACC     1151
Pro Val Pro Asp Phe Ser Phe Tyr Asp Arg Ser Ala Pro Ile Tyr Thr
        370                 375                 380

CAA CCT CGA TAT CTA CCA CCA TCA AAA ATG CTT GAT GCT GAT GTC ACA     1199
Gln Pro Arg Tyr Leu Pro Pro Ser Lys Met Leu Asp Ala Asp Val Thr
    385                 390                 395

GAT AGT GTC ATT GGT GAA GGT TGT GTG ATC AAG AAC TGT AAG ATT CAT     1247
Asp Ser Val Ile Gly Glu Gly Cys Val Ile Lys Asn Cys Lys Ile His
400                 405                 410                 415

CAT TCC GTG GTT GGA CTC AGA TCA TGC ATA TCA GAG GGA GCA ATT ATA     1295
His Ser Val Val Gly Leu Arg Ser Cys Ile Ser Glu Gly Ala Ile Ile
                420                 425                 430

GAA GAC TCA CTT TTG ATG GGG GCA GAT TAC TAT GAG ACT GAT GCT GAC     1343
Glu Asp Ser Leu Leu Met Gly Ala Asp Tyr Tyr Glu Thr Asp Ala Asp
            435                 440                 445

AGG AAG TTG CTG GCT GCA AAG GGC AGT GTC CCA ATT GGC ATC GGC AAG     1391
Arg Lys Leu Leu Ala Ala Lys Gly Ser Val Pro Ile Gly Ile Gly Lys
        450                 455                 460

AAT TGT CAC ATT AAA AGA GCC ATT ATC GAC AAG AAT GCC CGT ATA GGG     1439
Asn Cys His Ile Lys Arg Ala Ile Ile Asp Lys Asn Ala Arg Ile Gly
    465                 470                 475

GAC AAT GTG AAG ATC ATT AAC AAA GAC AAC GTT CAA GAA GCG GCT AGG     1487
Asp Asn Val Lys Ile Ile Asn Lys Asp Asn Val Gln Glu Ala Ala Arg
480                 485                 490                 495

GAA ACA GAT GGA TAC TTC ATC AAG AGT GGG ATT GTC ACC GTC ATC AAG     1535
```

```
Glu Thr Asp Gly Tyr Phe Ile Lys Ser Gly Ile Val Thr Val 1
                500                 505                 5

GAT GCT TTG ATT CCA AGT GGA ATC ATC ATC TGATGAGCTC                1575
Asp Ala Leu Ile Pro Ser Gly Ile Ile Ile
            515                 520
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 521 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
Met Ala Ala Ser Ile Gly Ala Leu Lys Ser Ser Pro Ser Ser Asn Asn
  1               5                  10                  15

Cys Ile Asn Glu Arg Arg Asn Asp Ser Thr Arg Ala Val Ser Ser Arg
             20                  25                  30

Asn Leu Ser Phe Ser Ser Ser His Leu Ala Gly Asp Lys Leu Met Pro
         35                  40                  45

Val Ser Ser Leu Arg Ser Gln Gly Val Arg Phe Asn Val Arg Arg Ser
     50                  55                  60

Pro Met Ile Val Ser Pro Lys Ala Val Ser Asp Ser Gln Asn Ser Gln
 65                  70                  75                  80

Thr Cys Leu Asp Pro Asp Ala Ser Arg Ser Val Leu Gly Ile Ile Leu
                 85                  90                  95

Gly Gly Gly Ala Gly Thr Arg Leu Tyr Pro Leu Thr Lys Lys Arg Ala
            100                 105                 110

Lys Pro Ala Val Pro Leu Gly Ala Asn Tyr Arg Leu Ile Asp Ile Pro
        115                 120                 125

Val Ser Asn Cys Leu Asn Ser Asn Ile Ser Lys Ile Tyr Val Leu Thr
    130                 135                 140

Gln Phe Asn Ser Ala Ser Leu Asn Arg His Leu Ser Arg Ala Tyr Ala
145                 150                 155                 160

Ser Asn Met Gly Gly Tyr Lys Asn Glu Gly Phe Val Glu Val Leu Ala
                165                 170                 175

Ala Gln Gln Ser Pro Glu Asn Pro Asp Trp Phe Gln Gly Thr Ala Asp
            180                 185                 190

Ala Val Arg Gln Tyr Leu Trp Leu Phe Glu Glu His Thr Val Leu Glu
        195                 200                 205

Tyr Leu Ile Leu Ala Gly Asp His Leu Tyr Arg Met Asp Tyr Glu Lys
    210                 215                 220

Phe Ile Gln Ala His Arg Glu Thr Asp Ala Asp Ile Thr Val Ala Ala
225                 230                 235                 240
```

```
Leu Pro Met Asp Glu Lys Arg Ala Thr Ala Phe Gly Leu Met Ly
                245                 250                 25

Asp Glu Glu Gly Arg Ile Ile Glu Phe Ala Glu Lys Pro Gln Gly Glu
            260                 265                 270

Gln Leu Gln Ala Met Lys Val Asp Thr Thr Ile Leu Gly Leu Asp Asp
        275                 280                 285

Lys Arg Ala Lys Glu Met Pro Phe Ile Ala Ser Met Gly Ile Tyr Val
    290                 295                 300

Ile Ser Lys Asp Val Met Leu Asn Leu Leu Arg Asp Lys Phe Pro Gly
305                 310                 315                 320

Ala Asn Asp Phe Gly Ser Glu Val Ile Pro Gly Ala Thr Ser Leu Gly
                325                 330                 335

Met Arg Val Gln Ala Tyr Leu Tyr Asp Gly Tyr Trp Glu Asp Ile Gly
            340                 345                 350

Thr Ile Glu Ala Phe Tyr Asn Ala Asn Leu Gly Ile Thr Lys Lys Pro
        355                 360                 365

Val Pro Asp Phe Ser Phe Tyr Asp Arg Ser Ala Pro Ile Tyr Thr Gln
    370                 375                 380

Pro Arg Tyr Leu Pro Pro Ser Lys Met Leu Asp Ala Asp Val Thr Asp
385                 390                 395                 400

Ser Val Ile Gly Glu Gly Cys Val Ile Lys Asn Cys Lys Ile His His
                405                 410                 415

Ser Val Val Gly Leu Arg Ser Cys Ile Ser Glu Gly Ala Ile Ile Glu
            420                 425                 430

Asp Ser Leu Leu Met Gly Ala Asp Tyr Tyr Glu Thr Asp Ala Asp Arg
        435                 440                 445

Lys Leu Leu Ala Ala Lys Gly Ser Val Pro Ile Gly Ile Gly Lys Asn
    450                 455                 460

Cys His Ile Lys Arg Ala Ile Ile Asp Lys Asn Ala Arg Ile Gly Asp
465                 470                 475                 480

Asn Val Lys Ile Ile Asn Lys Asp Asn Val Gln Glu Ala Ala Arg Glu
                485                 490                 495

Thr Asp Gly Tyr Phe Ile Lys Ser Gly Ile Val Thr Val Ile Lys Asp
            500                 505                 510

Ala Leu Ile Pro Ser Gly Ile Ile Ile
        515                 520
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1519 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1410

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
AAC AAG ATC AAA CCT GGG GTT GCT TAC TCT GTG ATC ACT ACT GAA AAT      48
Asn Lys Ile Lys Pro Gly Val Ala Tyr Ser Val Ile Thr Thr Glu Asn
  1               5                  10                  15

GAC ACA CAG ACT GTG TTC GTA GAT ATG CCA CGT CTT GAG AGA CGC CGG      96
Asp Thr Gln Thr Val Phe Val Asp Met Pro Arg Leu Glu Arg Arg Arg
             20                  25                  30

GCA AAT CCA AAG GAT GTG GCT GCA GTC ATA CTG GGA GGA GGA GAA GGG     144
Ala Asn Pro Lys Asp Val Ala Ala Val Ile Leu Gly Gly Gly Glu Gly
         35                  40                  45

ACC AAG TTA TTC CCA CTT ACA AGT AGA ACT GCA ACC CCT GCT GTT CCG     192
Thr Lys Leu Phe Pro Leu Thr Ser Arg Thr Ala Thr Pro Ala Val Pro
     50                  55                  60

GTT GGA GGA TGC TAC AGG CTA ATA GAC ATC CCA ATG AGC AAC TGT ATC     240
Val Gly Gly Cys Tyr Arg Leu Ile Asp Ile Pro Met Ser Asn Cys Ile
 65                  70                  75                  80

AAC AGT GCT ATT AAC AAG ATT TTT GTG CTG ACA CAG TAC AAT TCT GCT     288
Asn Ser Ala Ile Asn Lys Ile Phe Val Leu Thr Gln Tyr Asn Ser Ala
                 85                  90                  95

CCC CTG AAT CGT CAC ATT GCT CGA ACA TAT TTT GGC AAT GGT GTG AGC     336
Pro Leu Asn Arg His Ile Ala Arg Thr Tyr Phe Gly Asn Gly Val Ser
            100                 105                 110

TTT GGA GAT GGA TTT GTC GAG GTA CTA GCT GCA ACT CAG ACA CCC GGG     384
Phe Gly Asp Gly Phe Val Glu Val Leu Ala Ala Thr Gln Thr Pro Gly
        115                 120                 125

GAA GCA GGA AAA AAA TGG TTT CAA GGA ACA GCA GAT GCT GTT AGA AAA     432
Glu Ala Gly Lys Lys Trp Phe Gln Gly Thr Ala Asp Ala Val Arg Lys
    130                 135                 140

TTT ATA TGG GTT TTT GAG GAC GCT AAG AAC AAG AAT ATT GAA AAT ATC     480
Phe Ile Trp Val Phe Glu Asp Ala Lys Asn Lys Asn Ile Glu Asn Ile
145                 150                 155                 160

GTT GTA CTA TCT GGG GAT CAT CTT TAT AGG ATG GAT TAT ATG GAG TTG     528
Val Val Leu Ser Gly Asp His Leu Tyr Arg Met Asp Tyr Met Glu Leu
                165                 170                 175

GTG CAG AAC CAT ATT GAC AGG AAT GCT GAT ATT ACT CTT TCA TGT GCA     576
Val Gln Asn His Ile Asp Arg Asn Ala Asp Ile Thr Leu Ser Cys Ala
            180                 185                 190

CCA GCT GAG GAC AGC CGA GCA TCA GAT TTT GGG CTG GTC AAG ATT GAC     624
Pro Ala Glu Asp Ser Arg Ala Ser Asp Phe Gly Leu Val Lys Ile Asp
        195                 200                 205

AGC AGA GGC AGA GTA GTC CAG TTT GCT GAA AAA CCA AAA GGT TTT GAT     672
Ser Arg Gly Arg Val Val Gln Phe Ala Glu Lys Pro Lys Gly Phe Asp
    210                 215                 220
```

```
CTT AAA GCA ATG CAA GTA GAT ACT ACT CTT GTT GGA TTA TCT C
Leu Lys Ala Met Gln Val Asp Thr Thr Leu Val Gly Leu Ser F
225                 230                 235                 240

GAT GCG AAG AAA TCC CCC TAT ATT GCT TCA ATG GGA GTT TAT GTA TTC      768
Asp Ala Lys Lys Ser Pro Tyr Ile Ala Ser Met Gly Val Tyr Val Phe
                245                 250                 255

AAG ACA GAT GTA TTG TTG AAG CTC TTG AAA TGG AGC TAT CCC ACT TCT      816
Lys Thr Asp Val Leu Leu Lys Leu Leu Lys Trp Ser Tyr Pro Thr Ser
            260                 265                 270

AAT GAT TTT GGC TCT GAA ATT ATA CCA GCA GCT ATT GAC GAT TAC AAT      864
Asn Asp Phe Gly Ser Glu Ile Ile Pro Ala Ala Ile Asp Asp Tyr Asn
        275                 280                 285

GTC CAA GCA TAC ATT TTC AAA GAC TAT TGG GAA GAC ATT GGA ACA ATT      912
Val Gln Ala Tyr Ile Phe Lys Asp Tyr Trp Glu Asp Ile Gly Thr Ile
    290                 295                 300

AAA TCG TTT TAT AAT GCT AGC TTG GCA CTC ACA CAA GAG TTT CCA GAG      960
Lys Ser Phe Tyr Asn Ala Ser Leu Ala Leu Thr Gln Glu Phe Pro Glu
305                 310                 315                 320

TTC CAA TTT TAC GAT CCA AAA ACA CCT TTT TAC ACA TCT CCT AGG TTC     1008
Phe Gln Phe Tyr Asp Pro Lys Thr Pro Phe Tyr Thr Ser Pro Arg Phe
                325                 330                 335

CTT CCA CCA ACC AAG ATA GAC AAT TGC AAG ATT AAG GAT GCC ATA ATC     1056
Leu Pro Pro Thr Lys Ile Asp Asn Cys Lys Ile Lys Asp Ala Ile Ile
            340                 345                 350

TCT CAT GGA TGT TTC TTG CGA GAT TGT TCT GTG GAA CAC TCC ATA GTG     1104
Ser His Gly Cys Phe Leu Arg Asp Cys Ser Val Glu His Ser Ile Val
        355                 360                 365

GGT GAA AGA TCG CGC TTA GAT TGT GGT GTT GAA CTG AAG GAT ACT TTC     1152
Gly Glu Arg Ser Arg Leu Asp Cys Gly Val Glu Leu Lys Asp Thr Phe
    370                 375                 380

ATG ATG GGA GCA GAC TAC TAC CAA ACA GAA TCT GAG ATT GCC TCC CTG     1200
Met Met Gly Ala Asp Tyr Tyr Gln Thr Glu Ser Glu Ile Ala Ser Leu
385                 390                 395                 400

TTA GCA GAG GGG AAA GTA CCG ATT GGA ATT GGG GAA AAT ACA AAA ATA     1248
Leu Ala Glu Gly Lys Val Pro Ile Gly Ile Gly Glu Asn Thr Lys Ile
                405                 410                 415

AGG AAA TGT ATC ATT GAC AAG AAC GCA AAG ATA GGA AAG AAT GTT TCA     1296
Arg Lys Cys Ile Ile Asp Lys Asn Ala Lys Ile Gly Lys Asn Val Ser
            420                 425                 430

ATC ATA AAT AAA GAC GGT GTT CAA GAG GCA GAC CGA CCA GAG GAA GGA     1344
Ile Ile Asn Lys Asp Gly Val Gln Glu Ala Asp Arg Pro Glu Glu Gly
        435                 440                 445

TTC TAC ATA CGA TCA GGG ATA ATC ATT ATA TTA GAG AAA GCC ACA ATT     1392
Phe Tyr Ile Arg Ser Gly Ile Ile Ile Ile Leu Glu Lys Ala Thr Ile
    450                 455                 460

AGA GAT GGA ACA GTC ATC TGAACTAGGG AAGCACCTCT TGTTGAACTA            1440
Arg Asp Gly Thr Val Ile
465                 470
```

CTGGAGATCC AAATCTCAAC TTGAAGAAGG TCAAGGGTGA TCCTAGCAC

GACTCCCCGA AGGAAGCTT

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 470 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
Asn Lys Ile Lys Pro Gly Val Ala Tyr Ser Val Ile Thr Thr Glu Asn
  1               5                  10                  15

Asp Thr Gln Thr Val Phe Val Asp Met Pro Arg Leu Glu Arg Arg Arg
             20                  25                  30

Ala Asn Pro Lys Asp Val Ala Ala Val Ile Leu Gly Gly Gly Glu Gly
         35                  40                  45

Thr Lys Leu Phe Pro Leu Thr Ser Arg Thr Ala Thr Pro Ala Val Pro
     50                  55                  60

Val Gly Gly Cys Tyr Arg Leu Ile Asp Ile Pro Met Ser Asn Cys Ile
 65                  70                  75                  80

Asn Ser Ala Ile Asn Lys Ile Phe Val Leu Thr Gln Tyr Asn Ser Ala
                 85                  90                  95

Pro Leu Asn Arg His Ile Ala Arg Thr Tyr Phe Gly Asn Gly Val Ser
            100                 105                 110

Phe Gly Asp Gly Phe Val Glu Val Leu Ala Ala Thr Gln Thr Pro Gly
        115                 120                 125

Glu Ala Gly Lys Lys Trp Phe Gln Gly Thr Ala Asp Ala Val Arg Lys
    130                 135                 140

Phe Ile Trp Val Phe Glu Asp Ala Lys Asn Lys Asn Ile Glu Asn Ile
145                 150                 155                 160

Val Val Leu Ser Gly Asp His Leu Tyr Arg Met Asp Tyr Met Glu Leu
                165                 170                 175

Val Gln Asn His Ile Asp Arg Asn Ala Asp Ile Thr Leu Ser Cys Ala
            180                 185                 190

Pro Ala Glu Asp Ser Arg Ala Ser Asp Phe Gly Leu Val Lys Ile Asp
        195                 200                 205

Ser Arg Gly Arg Val Val Gln Phe Ala Glu Lys Pro Lys Gly Phe Asp
    210                 215                 220

Leu Lys Ala Met Gln Val Asp Thr Thr Leu Val Gly Leu Ser Pro Gln
225                 230                 235                 240

Asp Ala Lys Lys Ser Pro Tyr Ile Ala Ser Met Gly Val Tyr Val Phe
                245                 250                 255
```

Lys Thr Asp Val Leu Leu Lys Leu Leu Lys Trp Ser Tyr Pro :
260 265 270

Asn Asp Phe Gly Ser Glu Ile Ile Pro Ala Ala Ile Asp Asp Tyr Asn
275 280 285

Val Gln Ala Tyr Ile Phe Lys Asp Tyr Trp Glu Asp Ile Gly Thr Ile
290 295 300

Lys Ser Phe Tyr Asn Ala Ser Leu Ala Leu Thr Gln Glu Phe Pro Glu
305 310 315 320

Phe Gln Phe Tyr Asp Pro Lys Thr Pro Phe Tyr Thr Ser Pro Arg Phe
325 330 335

Leu Pro Pro Thr Lys Ile Asp Asn Cys Lys Ile Lys Asp Ala Ile Ile
340 345 350

Ser His Gly Cys Phe Leu Arg Asp Cys Ser Val Glu His Ser Ile Val
355 360 365

Gly Glu Arg Ser Arg Leu Asp Cys Gly Val Glu Leu Lys Asp Thr Phe
370 375 380

Met Met Gly Ala Asp Tyr Tyr Gln Thr Glu Ser Glu Ile Ala Ser Leu
385 390 395 400

Leu Ala Glu Gly Lys Val Pro Ile Gly Ile Gly Glu Asn Thr Lys Ile
405 410 415

Arg Lys Cys Ile Ile Asp Lys Asn Ala Lys Ile Gly Lys Asn Val Ser
420 425 430

Ile Ile Asn Lys Asp Gly Val Gln Glu Ala Asp Arg Pro Glu Glu Gly
435 440 445

Phe Tyr Ile Arg Ser Gly Ile Ile Ile Ile Leu Glu Lys Ala Thr Ile
450 455 460

Arg Asp Gly Thr Val Ile
465 470

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GTTGATAACA AGATCTGTTA ACCATGGCGG CTTCC 35

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS;
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CCAGTTAAAA CGGAGCTCAT CAGATGATGA TTC 33

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GTGTGAGAAC ATAAATCTTG GATATGTTAC 30

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GAATTCACAG GGCCATGGCT CTAGACCC 28

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AAGATCAAAC CTGCCATGGC TTACTCTGTG ATCACTACTG 40

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GGGAATTCAA GCTTGGATCC CGGGCCCCCC CCCCCCCCC 39

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GGGAATTCAA GCTTGGATCC CGGG 24

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CCTCTAGACA GTCGATCAGG AGCAGATGTA CG 32

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GGAGTTAGCC ATGGTTAGTT TAGAG 25

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

GGCCGAGCTC GTCAACGCCG TCTGCGATTT GTGC 34

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GATTTAGGTG ACACTATAG 19

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 42 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

AGAGAGATCT AGAACAATGG CTTCCTCTAT GCTCTCTTCC GC 42

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (synthetic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GGCCGAGCTC TAGATTATCG CTCCTGTTTA TGCCCTAAC 39

Claims:

1. A method for increasing the starch content of a plant which comprises altering said plant to increase the ADPglucose pyrophosphorylase activity in said plant.

2. A method of producing genetically transformed plants which have elevated starch content, comprising the steps of:

(a) inserting into the genome of a plant cell a recombinant, double-stranded DNA molecule comprising
   (i) a promoter which functions in plants to cause the production of an RNA sequence in the target plant tissues,
   (ii) a structural DNA sequence that causes the production of an RNA sequence which encodes a fusion polypeptide comprising an amino-terminal plastid transit peptide and an ADPglucose pyrophosphorylase enzyme,
   (iii) a 3' non-translated DNA sequence which functions in plant cells to cause transcriptional termination and the addition of polyadenylated nucleotides to the 3' end of the RNA sequence;

(b) obtaining transformed plant cells; and

(c) regenerating from the transformed plant cells genetically transformed plants which have an elevated starch content.

3. A method of claim 2 in which the ADPglucose pyrophosphorylase enzyme is deregulated.

4. A method of claim 3 in which the ADPglucose pyrophosphorylase enzyme is from bacteria.

5. A method of claim 3 in which the ADPglucose pyrophosphorylase enzyme is from plants or algae.

6. A recombinant, double-stranded DNA molecule comprising in sequence:

(a) a promoter which functions in plants to cause the production of an RNA sequence in the target plant tissues;

(b) a structural DNA sequence that causes the production of an RNA sequence which encodes a fusion polypeptide comprising an amino-terminal plastid transit peptide and an ADPglucose pyrophosphorylase enzyme; and

(c) a 3' non-translated region which functions in plant cells to cause transcriptional termination and the addition of polyadenylated nucleotides to the 3' end of the RNA sequence,

said promoter is heterologous with respect to said structural DNA.

7. A DNA molecule of claim 6 in which the ADPglucose pyrophosphorylase enzyme is deregulated.

8. A DNA molecule of claim 6 in which the plastid transit peptide is heterologous to the source of the ADPglucose pyrophosphorylase structural DNA.

9. A DNA molecule of claim 8 in which the ADPglucose pyrophosphorylase is from bacteria.

10. A plant cell comprising a recombinant, double-stranded DNA molecule comprising in sequence:

(a) a promoter which functions in plants to cause the production of an RNA sequence in target plant tissues;

(b) a structural DNA sequence that causes the production of an RNA sequence which encodes a fusion polypeptide comprising an amino-terminal plastid transit peptide and an ADPglucose pyrophosphorylase enzyme; and

(c) a 3' non-translated region which functions in plant cells to cause transcriptional termination and the addition of polyadenylated nucleotides to the 3' end of the RNA sequence

in which the DNA molecule is foreign to said plant cell.

11. A plant cell of claim 11 in which the ADPglucose pyrophosphorylase enzyme is deregulated.

12. A plant cell of claim 11 in which the promoter is heterologous with respect to the ADPglucose pyrophosphorylase structural DNA.

13. A plant cell of claim 12 in which the plastid transit peptide is heterologous to the source of the ADPglucose pyrophosphorylase structural DNA.

14. A plant cell of claim 13 in which the ADPglucose pyrophosphorylase is from bacteria.

15. A plant cell of claim 10 selected from the group consisting of corn, wheat, rice, carrot, onion, pea, tomato, potato and sweet potato, peanut, canola/oilseed rape, barley, sorghum, cassava, banana, soybeans, lettuce, apple and walnut.

16. A plant consisting of plant cells of claim 10.

17. A plant of claim 16 in which the ADPglucose pyrophosphorylase enzyme is deregulated.

18. A plant of claim 17 in which the promoter is heterologous to the source of the ADPglucose pyrophosphorylase structural DNA.

19. A plant of claim 17 in which the plastid transit peptide is heterologous to the source of the ADPglucose pyrophosphorylase structural DNA.

20. A plant of claim 18 in which the ADPglucose pyrophosphorylase is from bacteria.

21. A potato plant cell of claim 10.

22. A potato plant cell of claim 14.

23. A potato plant of claim 18.

24. A potato plant of claim 20.

25. A potato plant of claim 22 which is var. Russet-Burbank.

26. A potato plant of claim 24 which is var. Russet-Burbank.

27. A method of claim 1 in which said plant is potato.

28. A method of claim 2 in which said plant is potato.

29. A method of claim 3 in which said plant is potato.

30. A method of claim 4 in which said plant is potato.

31. A tomato plant cell of claim 10.

32. A tomato plant cell of claim 14.

33. A tomato plant of claim 16.

34. A tomato plant of claim 20.

35. A method of claim 1 in which said plant is tomato.

36. A method of claim 2 in which said plant is tomato.

37. A method of claim 3 in which said plant is tomato.

38. A method of claim 4 in which said plant is tomato.

1/15

```
DNA      ATGGTTAGTTTAGAGAAGAACGATCACTTAATGTTGGCGCGCCAGCTGCCATTGAAATCT
       1 ---------|---------|---------|---------|---------|---------|
Protein  M  V  S  L  E  K  N  D  H  L  M  L  A  R  Q  L  P  L  K  S

         GTTGCCCTGATACTGGCGGGAGGACGTGGTACCCGCCTGAAGGATTTAACCAATAAGCGA
      61 ---------|---------|---------|---------|---------|---------|
         V  A  L  I  L  A  G  G  R  G  T  R  L  K  D  L  T  N  K  R

         GCAAAACCGGCCGTACACTTCGGCGGTAAGTTCCGCATTATCGACTTTGCGCTGTCTAAC
     121 ---------|---------|---------|---------|---------|---------|
         A  K  P  A  V  H  F  G  G  K  F  R  I  I  D  F  A  L  S  N

         TGCATCAACTCCGGGATCCGTCGTATGGGCGTGATCACCCAGTACCAGTCCCACACTCTG
     181 ---------|---------|---------|---------|---------|---------|
         C  I  N  S  G  I  R  R  M  G  V  I  T  Q  Y  Q  S  H  T  L

         GTGCAGCACATTCAGCGCGGCTGGTCATTCTTCAATGAAGAAATGAACGAGTTTGTCGAT
     241 ---------|---------|---------|---------|---------|---------|
         V  Q  H  I  Q  R  G  W  S  F  F  N  E  E  M  N  E  F  V  D

         CTGCTGCCAGCACAGCAGAGAATGAAAGGGGAAAACTGGTATCGCGGCACCGCAGATGCG
     301 ---------|---------|---------|---------|---------|---------|
         L  L  P  A  Q  Q  R  M  K  G  E  N  W  Y  R  G  T  A  D  A

         GTCACCCAAAACCTCGACATTATCCGTCGTTATAAAGCGGAATACGTGGTGATCCTGGCG
     361 ---------|---------|---------|---------|---------|---------|
         V  T  Q  N  L  D  I  I  R  R  Y  K  A  E  Y  V  V  I  L  A

         GGCGACCATATCTACAAGCAAGACTACTCGCGTATGCTTATCGATCACGTCGAAAAAGGT
     421 ---------|---------|---------|---------|---------|---------|
         G  D  H  I  Y  K  Q  D  Y  S  R  M  L  I  D  H  V  E  K  G

         GTACGTTGTACCGTTGTTTGTATGCCAGTACCGATTGAAGAAGCCTCCGCATTTGGCGTT
     481 ---------|---------|---------|---------|---------|---------|
         V  R  C  T  V  V  C  M  P  V  P  I  E  E  A  S  A  F  G  V

         ATGGCGGTTGATGAGAACGATAAAACTATCGAATTCGTGGAAAAACCTGCTAACCCGCCG
     541 ---------|---------|---------|---------|---------|---------|
         M  A  V  D  E  N  D  K  T  I  E  F  V  E  K  P  A  N  P  P

         TCAATGCCGAACGATCCGAGCAAATCTCTGGCGAGTATGGGTATCTACGTCTTTGACGCC
     601 ---------|---------|---------|---------|---------|---------|
         S  M  P  N  D  P  S  K  S  L  A  S  M  G  I  Y  V  F  D  A

         GACTATCTGTATGAACTGCTGGAAGAAGACGATCGCGATGAGAACTCCAGCCACGACTTT
     661 ---------|---------|---------|---------|---------|---------|
         D  Y  L  Y  E  L  L  E  E  D  D  R  D  E  N  S  S  H  D  F
```

FIG. 1A

2/15

```
     GGCAAAGATTTGATTCCCAAGATCACCGAAGCCGGTCTGGCCTATGCGCACCCGTTCCCG
 721 ---------|---------|---------|---------|---------|---------|
     G  K  D  L  I  P  K  I  T  E  A  G  L  A  Y  A  H  P  F  P

     CTCTCTTGCGTACAATCCGACCCGGATGCCGAGCCGTACTGGCGCGATGTGGGTACGCTG
 781 ---------|---------|---------|---------|---------|---------|
     L  S  C  V  Q  S  D  P  D  A  E  P  Y  W  R  D  V  G  T  L

     GAAGCTTACTGGAAAGCGAACCTCGATCTGGCCTCTGTGGTGCCGAAACTGGATATGTAC
 841 ---------|---------|---------|---------|---------|---------|
     E  A  Y  W  K  A  N  L  D  L  A  S  V  V  P  K  L  D  M  Y

     GATCGCAATTGGCCAATTCGCACCTACAATGAATCATTACCGCCAGCGAAATTCGTGCAG
 901 ---------|---------|---------|---------|---------|---------|
     D  R  N  W  P  I  R  T  Y  N  E  S  L  P  P  A  K  F  V  Q

     GATCGCTCCGGTAGCCACGGGATGACCCTTAACTCACTGGTTTCCGGCGGTTGTGTGATC
 961 ---------|---------|---------|---------|---------|---------|
     D  R  S  G  S  H  G  M  T  L  N  S  L  V  S  G  G  C  V  I

     TCCGGTTCGGTGGTGGTGCAGTCCGTTCTGTTCTCGCGCGTTCGCGTGAATTCATTCTGC
1021 ---------|---------|---------|---------|---------|---------|
     S  G  S  V  V  V  Q  S  V  L  F  S  R  V  R  V  N  S  F  C

     AACATTGATTCCGCCGTATTGTTACCGGAAGTATGGGTAGGTCGCTCGTGCCGTCTGCGC
1081 ---------|---------|---------|---------|---------|---------|
     N  I  D  S  A  V  L  L  P  E  V  W  V  G  R  S  C  R  L  R

     CGCTGCGTCATCGATCGTGCTTGTGTTATTCCGGAAGGCATGGTGATTGGTGAAAACGCA
1141 ---------|---------|---------|---------|---------|---------|
     R  C  V  I  D  R  A  C  V  I  P  E  G  M  V  I  G  E  N  A

     GAGGAAGATGCACGTCGTTTCTATCGTTCAGAAGAAGGCATCGTGCTGGTAACGCGCGAA
1201 ---------|---------|---------|---------|---------|---------|
     E  E  D  A  R  R  F  Y  R  S  E  E  G  I  V  L  V  T  R  E

     ATGCTACGGAAGTTAGGGCATAAACAGGAGCGATAA
1261 ---------|---------|---------|------
     M  L  R  K  L  G  H  K  Q  E  R  *
```

FIG. 1B

```
DNA      ATGGTTAGTTTAGAGAAGAACGATCACTTAATGTTGGCGCGCCAGCTGCCATTGAAATCT
       1 ---------|---------|---------|---------|---------|---------|
Protein  M  V  S  L  E  K  N  D  H  L  M  L  A  R  Q  L  P  L  K  S

         GTTGCCCTGATACTGGCGGGAGGACGTGGTACCCGCCTGAAGGATTTAACCAATAAGCGA
      61 ---------|---------|---------|---------|---------|---------|
         V  A  L  I  L  A  G  G  R  G  T  R  L  K  D  L  T  N  K  R

         GCAAAACCGGCCGTACACTTCGGCGGTAAGTTCCGCATTATCGACTTTGCGCTGTCTAAC
     121 ---------|---------|---------|---------|---------|---------|
         A  K  P  A  V  H  F  G  G  K  F  R  I  I  D  F  A  L  S  N

         TGCATCAACTCCGGGATCCGTCGTATGGGCGTGATCACCCAGTACCAGTCCCACACTCTG
     181 ---------|---------|---------|---------|---------|---------|
         C  I  N  S  G  I  R  R  M  G  V  I  T  Q  Y  Q  S  H  T  L

         GTGCAGCACATTCAGCGCGGCTGGTCATTCTTCAATGAAGAAATGAACGAGTTTGTCGAT
     241 ---------|---------|---------|---------|---------|---------|
         V  Q  H  I  Q  R  G  W  S  F  F  N  E  E  M  N  E  F  V  D

         CTGCTGCCAGCACAGCAGAGAATGAAAGGGGAAAACTGGTATCGCGGCACCGCAGATGCG
     301 ---------|---------|---------|---------|---------|---------|
         L  L  P  A  Q  Q  R  M  K  G  E  N  W  Y  R  G  T  A  D  A

         GTCACCCAAAACCTCGACATTATCCGTCGTTATAAAGCGGAATACGTGGTGATCCTGGCG
     361 ---------|---------|---------|---------|---------|---------|
         V  T  Q  N  L  D  I  I  R  R  Y  K  A  E  Y  V  V  I  L  A

         GGCGACCATATCTACAAGCAAGACTACTCGCGTATGCTTATCGATCACGTCGAAAAAGGT
     421 ---------|---------|---------|---------|---------|---------|
         G  D  H  I  Y  K  Q  D  Y  S  R  M  L  I  D  H  V  E  K  G

         GTACGTTGTACCGTTGTTTGTATGCCAGTACCGATTGAAGAAGCCTCCGCATTTGGCGTT
     481 ---------|---------|---------|---------|---------|---------|
         V  R  C  T  V  V  C  M  P  V  P  I  E  E  A  S  A  F  G  V

         ATGGCGGTTGATGAGAACGATAAAACTATCGAATTCGTGGAAAAACCTGCTAACCCGCCG
     541 ---------|---------|---------|---------|---------|---------|
         M  A  V  D  E  N  D  K  T  I  E  F  V  E  K  P  A  N  P  P

         TCAATGCCGAACGATCCGAGCAAATCTCTGGCGAGTATGGGTATCTACGTCTTTGACGCC
     601 ---------|---------|---------|---------|---------|---------|
         S  M  P  N  D  P  S  K  S  L  A  S  M  G  I  Y  V  F  D  A

         GACTATCTGTATGAACTGCTGGAAGAAGACGATCGCGATGAGAACTCCAGCCACGACTTT
     661 ---------|---------|---------|---------|---------|---------|
         D  Y  L  Y  E  L  L  E  E  D  D  R  D  E  N  S  S  H  D  F

         GGCAAAGATTTGATTCCCAAGATCACCGAAGCCGGTCTGGCCTATGCGCACCCGTTCCCG
     721 ---------|---------|---------|---------|---------|---------|
         G  K  D  L  I  P  K  I  T  E  A  G  L  A  Y  A  H  P  F  P
```

FIG 2A

```
     CTCTCTTGCGTACAATCCGACCCGGATGCCGAGCCGTACTGGCGCGATGTGGGTACGCTG
 781 ----------|----------|----------|----------|----------|----------|
     L  S  C  V  Q  S  D  P  D  A  E  P  Y  W  R  D  V  G  T  L

     GAAGCTTACTGGAAAGCGAACCTCGATCTGGCCTCTGTGGTGCCGGAACTGGATATGTAC
 841 ----------|----------|----------|----------|----------|----------|
     E  A  Y  W  K  A  N  L  D  L  A  S  V  V  P  E  L  D  M  Y

     GATCGCAATTGGCCAATTCGCACCTACAATGAATCATTACCGCCAGCGAAATTCGTGCAG
 901 ----------|----------|----------|----------|----------|----------|
     D  R  N  W  P  I  R  T  Y  N  E  S  L  P  P  A  K  F  V  Q

     GATCGCTCCGGTAGCCACGGGATGACCCTTAACTCACTGGTTTCCGACGGTTGTGTGATC
 961 ----------|----------|----------|----------|----------|----------|
     D  R  S  G  S  H  G  M  T  L  N  S  L  V  S  D  G  C  V  I

     TCCGGTTCGGTGGTGGTGCAGTCCGTTCTGTTCTCGCGCGTTCGCGTGAATTCATTCTGC
1021 ----------|----------|----------|----------|----------|----------|
     S  G  S  V  V  V  Q  S  V  L  F  S  R  V  R  V  N  S  F  C

     AACATTGATTCCGCCGTATTGTTACCGGAAGTATGGGTAGGTCGCTCGTGCCGTCTGCGC
1081 ----------|----------|----------|----------|----------|----------|
     N  I  D  S  A  V  L  L  P  E  V  W  V  G  R  S  C  R  L  R

     CGCTGCGTCATCGATCGTGCTTGTGTTATTCCGGAAGGCATGGTGATTGGTGAAAACGCA
1141 ----------|----------|----------|----------|----------|----------|
     R  C  V  I  D  R  A  C  V  I  P  E  G  M  V  I  G  E  N  A

     GAGGAAGATGCACGTCGTTTCTATCGTTCAGAAGAAGGCATCGTGCTGGTAACGCGCGAA
1201 ----------|----------|----------|----------|----------|----------|
     E  E  D  A  R  R  F  Y  R  S  E  E  G  I  V  L  V  T  R  E

     ATGCTACGGAAGTTAGGGCATAAACAGGAGCGATAA
1261 ----------|----------|----------|------
     M  L  R  K  L  G  H  K  Q  E  R  *
```

FIG. 2B

```
H
i
n
d
I
I
I
  aagcttgttctcattgttgttatcattatatatagatgaccaaagcactagaccaaacct
1 ---------|---------|---------|---------|---------|---------| 60

   cagtcacacaaagagtaaagaagaacaatggcttcctctatgctctcttccgctactatg
61 ---------|---------|---------|---------|---------|---------| 120
                                 M  A  S  S  M  L  S  S  A  T  M

    gttgcctctccggctcaggccactatggtcgctcctttcaacggacttaagtcctccgct
121 ---------|---------|---------|---------|---------|---------| 180
    V  A  S  P  A  Q  A  T  M  V  A  P  F  N  G  L  K  S  S  A

    gccttcccagccacccgcaaggctaacaacgacattacttccatcacaagcaacggcgga
181 ---------|---------|---------|---------|---------|---------| 240
     A  F  P  A  T  R  K  A  N  N  D  I  T  S  I  T  S  N  G  G

    agagttaactgcatgcaggtgtggcctccgattggaaagaagaagtttgagactctctct
241 ---------|---------|---------|---------|---------|---------| 300
     R  V  N  C  M  Q  V  W  P  P  I  G  K  K  K  F  E  T  L  S

                                                       N
                                                       c
                                                       o
                                                       I
    taccttcctgaccttaccgattccggtggtcgcgtcaactgcatgcaggccatgg
301 ---------|---------|---------|---------|---------|----- 355
     Y  L  P  D  L  T  D  S  G  G  R  V  N  C  M  Q  A  M
```

FIG. 3

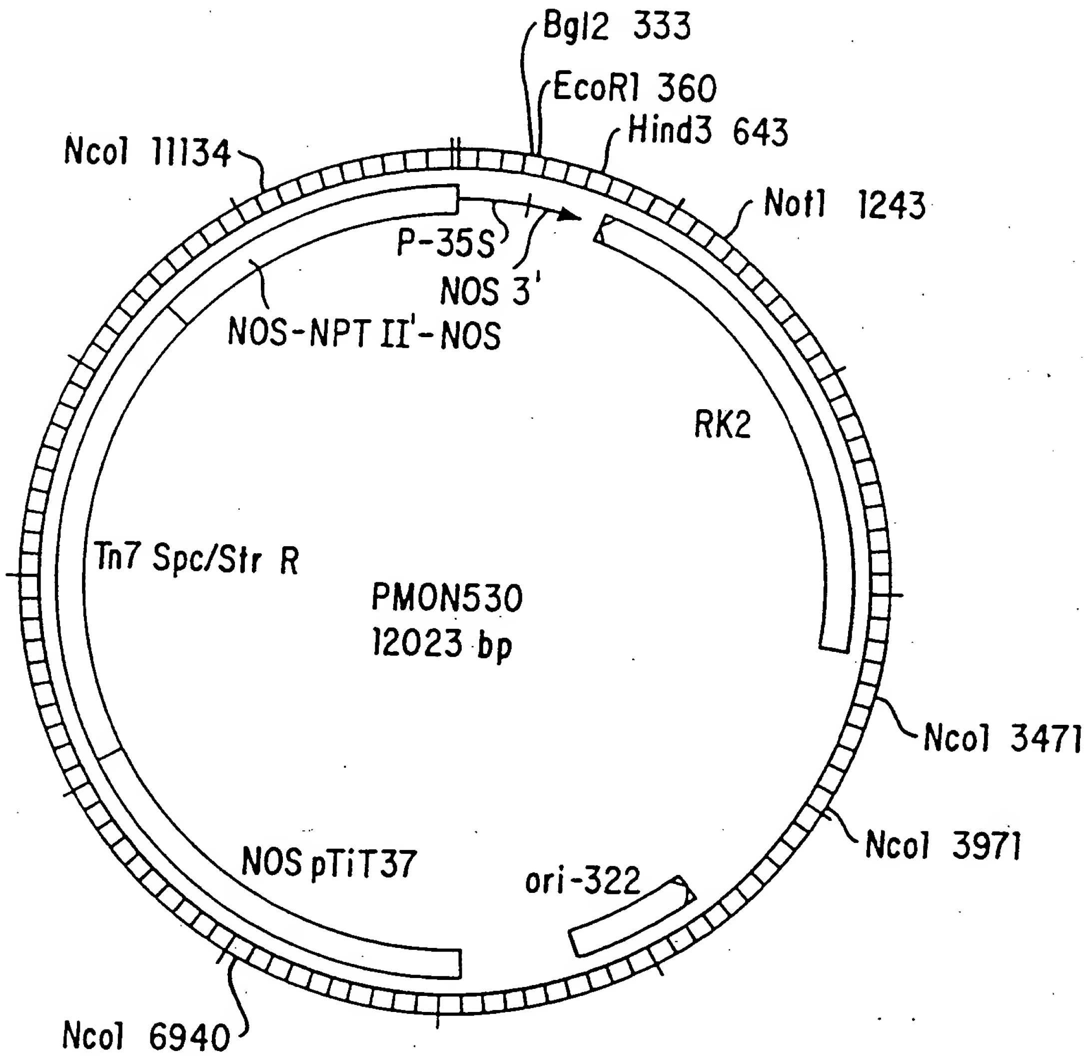

Bgl2 333
EcoR1 360
Hind3 643
Not1 1243
NcoI 11134
P-35S
NOS 3'
NOS-NPT II'-NOS
RK2
Tn7 Spc/Str R
PMON530
12023 bp
NcoI 3471
NcoI 3971
NOS pTiT37
ori-322
NcoI 6940

FIG. 4

```
  1 CCATGGCGGCTTCCATTGGAGCCTTAAAATCTTCACCTTCTTCTAACAATTGCATCAATG   60
      MetAlaAlaSerIleGlyAlaLeuLysSerSerProSerSerAsnAsnCysIleAsnG
 61 AGAGAAGAAATGATTCTACACGTGCTGTATCCAGCAGAAATCTCTCATTTTCGTCTTCTC  120
    luArgArgAsnAspSerThrArgAlaValSerSerArgAsnLeuSerPheSerSerSerH
121 ATCTCGCCGGAGACAAGTTGATGCCTGTATCGTCCTTACGTTCCCAAGGAGTCCGATTCA  180
    isLeuAlaGlyAspLysLeuMetProValSerSerLeuArgSerGlnGlyValArgPheA
181 ATGTGAGAAGAAGTCCAATGATTGTGTCGCCAAAGGCTGTTTCTGATTCGCAGAATTCAC  240
    snValArgArgSerProMetIleValSerProLysAlaValSerAspSerGlnAsnSerG
241 AGACATGTCTAGACCCAGATGCTAGCCGGAGTGTTTTGGGAATTATTCTTGGAGGTGGAG  300
    lnThrCysLeuAspProAspAlaSerArgSerValLeuGlyIleIleLeuGlyGlyGlyA
301 CTGGGACCCGACTTTATCCTCTAACTAAAAAAAGAGCAAAGCCAGCTGTTCCACTTGGAG  360
    laGlyThrArgLeuTyrProLeuThrLysLysArgAlaLysProAlaValProLeuGlyA
361 CAAATTATCGTCTGATTGACATTCCTGTAAGCAACTGCTTGAACAGTAATATATCCAAGA  420
    laAsnTyrArgLeuIleAspIleProValSerAsnCysLeuAsnSerAsnIleSerLysI
421 TTTATGTTCTCACACAATTCAACTCTGCCTCTCTGAATCGCCACCTTTCACGAGCATATG  480
    leTyrValLeuThrGlnPheAsnSerAlaSerLeuAsnArgHisLeuSerArgAlaTyrA
481 CTAGCAACATGGGAGGATACAAAAACGAGGGCTTTGTGGAAGTTCTTGCTGCTCAACAAA  540
    laSerAsnMetGlyGlyTyrLysAsnGluGlyPheValGluValLeuAlaAlaGlnGlnS
541 GTCCAGAGAACCCCGATTGGTTCCAGGGCACGGCTGATGCTGTCAGACAATATCTGTGGT  600
    erProGluAsnProAspTrpPheGlnGlyThrAlaAspAlaValArgGlnTyrLeuTrpL
601 TGTTTGAGGAGCATACTGTTCTTGAATACCTTATACTTGCTGGAGATCATCTGTATCGAA  660
    euPheGluGluHisThrValLeuGluTyrLeuIleLeuAlaGlyAspHisLeuTyrArgM
661 TGGATTATGAAAAGTTTATTCAAGCCCACAGAGAAACAGATGCTGATATTACCGTTGCCG  720
    etAspTyrGluLysPheIleGlnAlaHisArgGluThrAspAlaAspIleThrValAlaA
721 CACTGCCAATGGACGAGAAGCGTGCCACTGCATTCGGTCTCATGAAGATTGACGAAGAAG  780
    laLeuProMetAspGluLysArgAlaThrAlaPheGlyLeuMetLysIleAspGluGluG
781 GACGCATTATTGAATTTGCAGAGAAACCGCAAGGAGAGCAATTGCAAGCAATGAAAGTGG  840
    lyArgIleIleGluPheAlaGluLysProGlnGlyGluGlnLeuGlnAlaMetLysValA
```

FIG.5A

```
841  ATACTACCATTTTAGGTCTTGATGACAAGAGAGCTAAAGAAATGCCTTTCATTGCCAGTA  900
     spThrThrIleLeuGlyLeuAspAspLysArgAlaLysGluMetProPheIleAlaSerM
901  TGGGTATATATGTCATTAGCAAAGACGTGATGTTAAACCTACTTCGTGACAAGTTCCCTG  960
     etGlyIleTyrValIleSerLysAspValMetLeuAsnLeuLeuArgAspLysPheProG
961  GGGCCAATGATTTTGGTAGTGAAGTTATTCCTGGTGCAACTTCACTTGGGATGAGAGTGC 1020
     lyAlaAsnAspPheGlySerGluValIleProGlyAlaThrSerLeuGlyMetArgValG
1021 AAGCTTATTTATATGATGGGTACTGGGAAGATATTGGTACCATTGAAGCTTTCTACAATG 1080
     lnAlaTyrLeuTyrAspGlyTyrTrpGluAspIleGlyThrIleGluAlaPheTyrAsnA
1081 CCAATTTGGGCATTACAAAAAAGCCGGTGCCAGATTTTAGCTTTTACGACCGATCAGCCC 1140
     laAsnLeuGlyIleThrLysLysProValProAspPheSerPheTyrAspArgSerAlaP
1141 CAATCTACACCCAACCTCGATATCTACCACCATCAAAAATGCTTGATGCTGATGTCACAG 1200
     roIleTyrThrGlnProArgTyrLeuProProSerLysMetLeuAspAlaAspValThrA
1201 ATAGTGTCATTGGTGAAGGTTGTGTGATCAAGAACTGTAAGATTCATCATTCCGTGGTTG 1260
     spSerValIleGlyGluGlyCysValIleLysAsnCysLysIleHisHisSerValValG
1261 GACTCAGATCATGCATATCAGAGGGAGCAATTATAGAAGACTCACTTTTGATGGGGGCAG 1320
     lyLeuArgSerCysIleSerGluGlyAlaIleIleGluAspSerLeuLeuMetGlyAlaA
1321 ATTACTATGAGACTGATGCTGACAGGAAGTTGCTGGCTGCAAAGGGCAGTGTCCCAATTG 1380
     spTyrTyrGluThrAspAlaAspArgLysLeuLeuAlaAlaLysGlySerValProIleG
1381 GCATCGGCAAGAATTGTCACATTAAAAGAGCCATTATCGACAAGAATGCCCGTATAGGGG 1440
     lyIleGlyLysAsnCysHisIleLysArgAlaIleIleAspLysAsnAlaArgIleGlyA
1441 ACAATGTGAAGATCATTAACAAAGACAACGTTCAAGAAGCGGCTAGGGAAACAGATGGAT 1500
     spAsnValLysIleIleAsnLysAspAsnValGlnGluAlaAlaArgGluThrAspGlyT
1501 ACTTCATCAAGAGTGGGATTGTCACCGTCATCAAGGATGCTTTGATTCCAAGTGGAATCA 1560
     yrPheIleLysSerGlyIleValThrValIleLysAspAlaLeuIleProSerGlyIleI
1561 TCATCTGATGAGCTC  1575
     leIleEndEnd
```

FIG.5B

```
1    AACAAGATCAAACCTGGGGTTGCTTACTCTGTGATCACTACTGAAAATGACACACAGACT   60
     AsnLysIleLysProGlyValAlaTyrSerValIleThrThrGluAsnAspThrGlnThr
61   GTGTTCGTAGATATGCCACGTCTTGAGAGACGCCGGGCAAATCCAAAGGATGTGGCTGCA  120
     ValPheValAspMetProArgLeuGluArgArgArgAlaAsnProLysAspValAlaAla
121  GTCATACTGGGAGGAGGAGAAGGGACCAAGTTATTCCCACTTACAAGTAGAACTGCAACC  180
     ValIleLeuGlyGlyGlyGluGlyThrLysLeuPheProLeuThrSerArgThrAlaThr
181  CCTGCTGTTCCGGTTGGAGGATGCTACAGGCTAATAGACATCCCAATGAGCAACTGTATC  240
     ProAlaValProValGlyGlyCysTyrArgLeuIleAspIleProMetSerAsnCysIle
241  AACAGTGCTATTAACAAGATTTTTGTGCTGACACAGTACAATTCTGCTCCCCTGAATCGT  300
     AsnSerAlaIleAsnLysIlePheValLeuThrGlnTyrAsnSerAlaProLeuAsnArg
301  CACATTGCTCGAACATATTTTGGCAATGGTGTGAGCTTTGGAGATGGATTTGTCGAGGTA  360
     HisIleAlaArgThrTyrPheGlyAsnGlyValSerPheGlyAspGlyPheValGluVal
361  CTAGCTGCAACTCAGACACCCGGGGAAGCAGGAAAAAAATGGTTTCAAGGAACAGCAGAT  420
     LeuAlaAlaThrGlnThrProGlyGluAlaGlyLysLysTrpPheGlnGlyThrAlaAsp
421  GCTGTTAGAAAATTTATATGGGTTTTTGAGGACGCTAAGAACAAGAATATTGAAAATATC  480
     AlaValArgLysPheIleTrpValPheGluAspAlaLysAsnLysAsnIleGluAsnIle
481  GTTGTACTATCTGGGGATCATCTTTATAGGATGGATTATATGGAGTTGGTGCAGAACCAT  540
     ValValLeuSerGlyAspHisLeuTyrArgMetAspTyrMetGluLeuValGlnAsnHis
541  ATTGACAGGAATGCTGATATTACTCTTTCATGTGCACCAGCTGAGGACAGCCGAGCATCA  600
     IleAspArgAsnAlaAspIleThrLeuSerCysAlaProAlaGluAspSerArgAlaSer
601  GATTTTGGGCTGGTCAAGATTGACAGCAGAGGCAGAGTAGTCCAGTTTGCTGAAAAACCA  660
     AspPheGlyLeuValLysIleAspSerArgGlyArgValValGlnPheAlaGluLysPro
661  AAAGGTTTTGATCTTAAAGCAATGCAAGTAGATACTACTCTTGTTGGATTATCTCCACAA  720
     LysGlyPheAspLeuLysAlaMetGlnValAspThrThrLeuValGlyLeuSerProGln
```

FIG.6A

721 GATGCGAAGAAATCCCCCTATATTGCTTCAATGGGAGTTTATGTATTCAAGACAGATGTA 780
AspAlaLysLysSerProTyrIleAlaSerMetGlyValTyrValPheLysThrAspVal
781 TTGTTGAAGCTCTTGAAATGGAGCTATCCCACTTCTAATGATTTTGGCTCTGAAATTATA 840
LeuLeuLysLeuLeuLysTrpSerTyrProThrSerAsnAspPheGlySerGluIleIle
841 CCAGCAGCTATTGACGATTACAATGTCCAAGCATACATTTTCAAAGACTATTGGGAAGAC 900
ProAlaAlaIleAspAspTyrAsnValGlnAlaTyrIlePheLysAspTyrTrpGluAsp
901 ATTGGAACAATTAAATCGTTTTATAATGCTAGCTTGGCACTCACACAAGAGTTTCCAGAG 960
IleGlyThrIleLysSerPheTyrAsnAlaSerLeuAlaLeuThrGlnGluPheProGlu
961 TTCCAATTTTACGATCCAAAAACACCTTTTTACACATCTCCTAGGTTCCTTCCACCAACC 1020
PheGlnPheTyrAspProLysThrProPheTyrThrSerProArgPheLeuProProThr
1021 AAGATAGACAATTGCAAGATTAAGGATGCCATAATCTCTCATGGATGTTTCTTGCGAGAT 1080
LysIleAspAsnCysLysIleLysAspAlaIleIleSerHisGlyCysPheLeuArgAsp
1081 TGTTCTGTGGAACACTCCATAGTGGGTGAAAGATCGCGCTTAGATTGTGGTGTTGAACTG 1140
CysSerValGluHisSerIleValGlyGluArgSerArgLeuAspCysGlyValGluLeu
1141 AAGGATACTTTCATGATGGGAGCAGACTACTACCAAACAGAATCTGAGATTGCCTCCCTG 1200
LysAspThrPheMetMetGlyAlaAspTyrTyrGlnThrGluSerGluIleAlaSerLeu
1201 TTAGCAGAGGGGAAAGTACCGATTGGAATTGGGGAAAATACAAAAATAAGGAAATGTATC 1260
LeuAlaGluGlyLysValProIleGlyIleGlyGluAsnThrLysIleArgLysCysIle
1261 ATTGACAAGAACGCAAAGATAGGAAAGAATGTTTCAATCATAAATAAAGACGGTGTTCAA 1320
IleAspLysAsnAlaLysIleGlyLysAsnValSerIleIleAsnLysAspGlyValGln
1321 GAGGCAGACCGACCAGAGGAAGGATTCTACATACGATCAGGGATAATCATTATATTAGAG 1380
GluAlaAspArgProGluGluGlyPheTyrIleArgSerGlyIleIleIleIleLeuGlu
1381 AAAGCCACAATTAGAGATGGAACAGTCATCTGAACTAGGGAAGCACCTCTTGTTGAACTA 1440
LysAlaThrIleArgAspGlyThrValIleEnd
1441 CTGGAGATCCAAATCTCAACTTGAAGAAGGTCAAGGGTGATCCTAGCACGTTCACCAGTT 1500
1501 GACTCCCCGAAGGAAGCTT 1519

FIG.6B

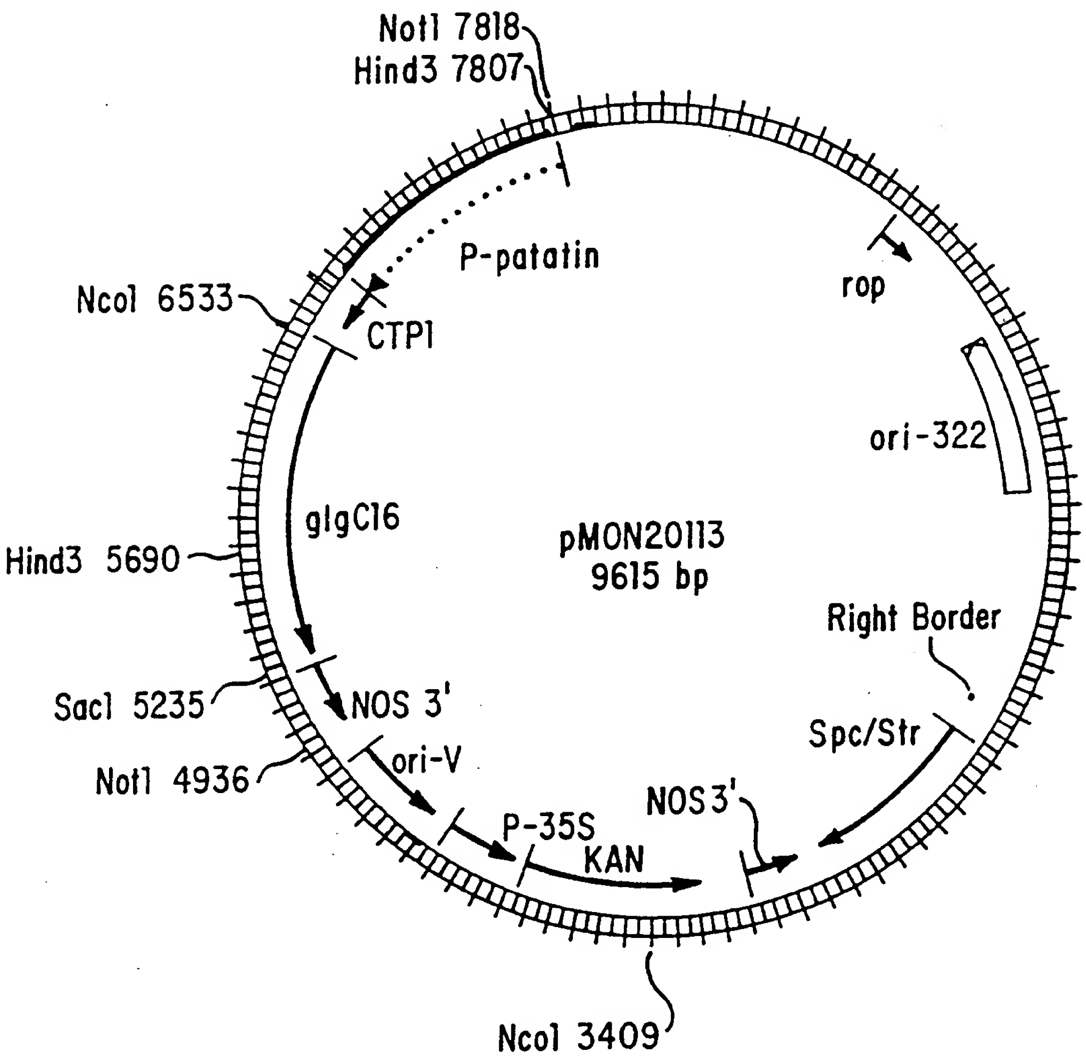

NotI 7818
Hind3 7807
P-patatin
rop
NcoI 6533
CTP1
ori-322
glgC16
pMON20113
9615 bp
Hind3 5690
Right Border
SacI 5235
NOS 3'
ori-V
Spc/Str
NotI 4936
NOS3'
P-35S
KAN
NcoI 3409

FIG. 7

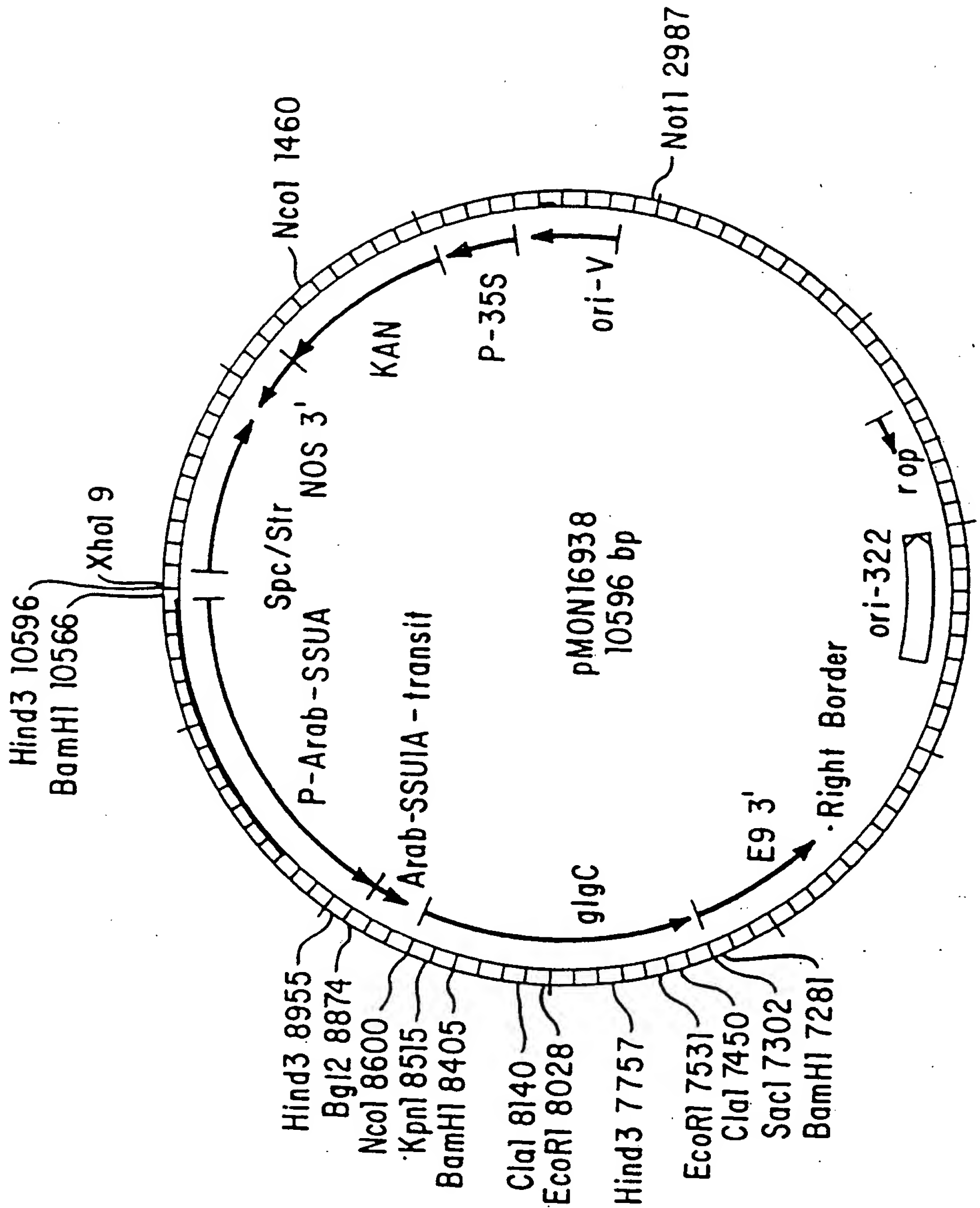

Hind3 10596
BamHI 10566
XhoI 9
NcoI 1460
NotI 2987
ori-V
P-35S
KAN
NOS 3'
Spc/Str
P-Arab-SSUA
Arab-SSUIA-transit
pMON16938
10596 bp
rop
ori-322
Right Border
E9 3'
glgC
Hind3 8955
BglI2 8874
NcoI 8600
KpnI 8515
BamHI 8405
ClaI 8140
EcoRI 8028
Hind3 7757
EcoRI 7531
ClaI 7450
SacI 7302
BamHI 7281

FIG. 8

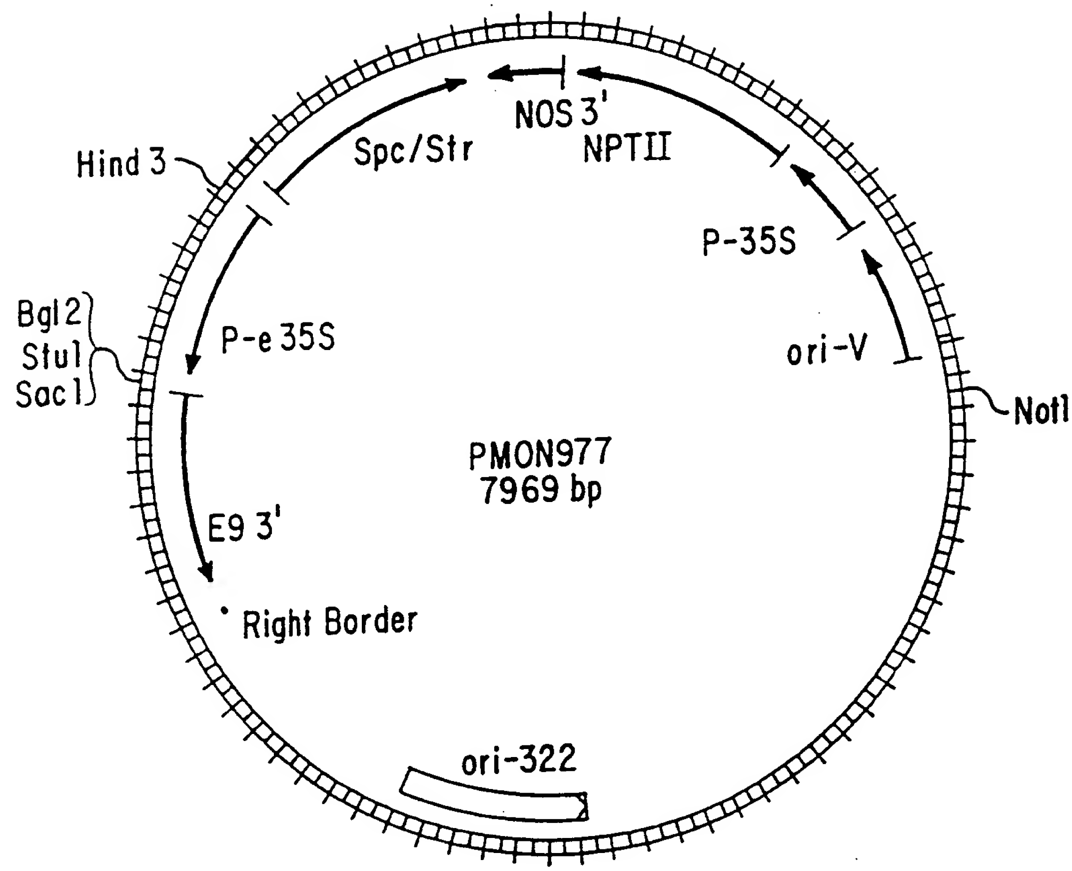

NOS 3'
NPTII
Spc/Str
Hind 3
P-35S
Bgl 2
Stu1
Sac1
P-e 35S
ori-V
NotI
PMON977
7969 bp
E9 3'
Right Border
ori-322

FIG. 9

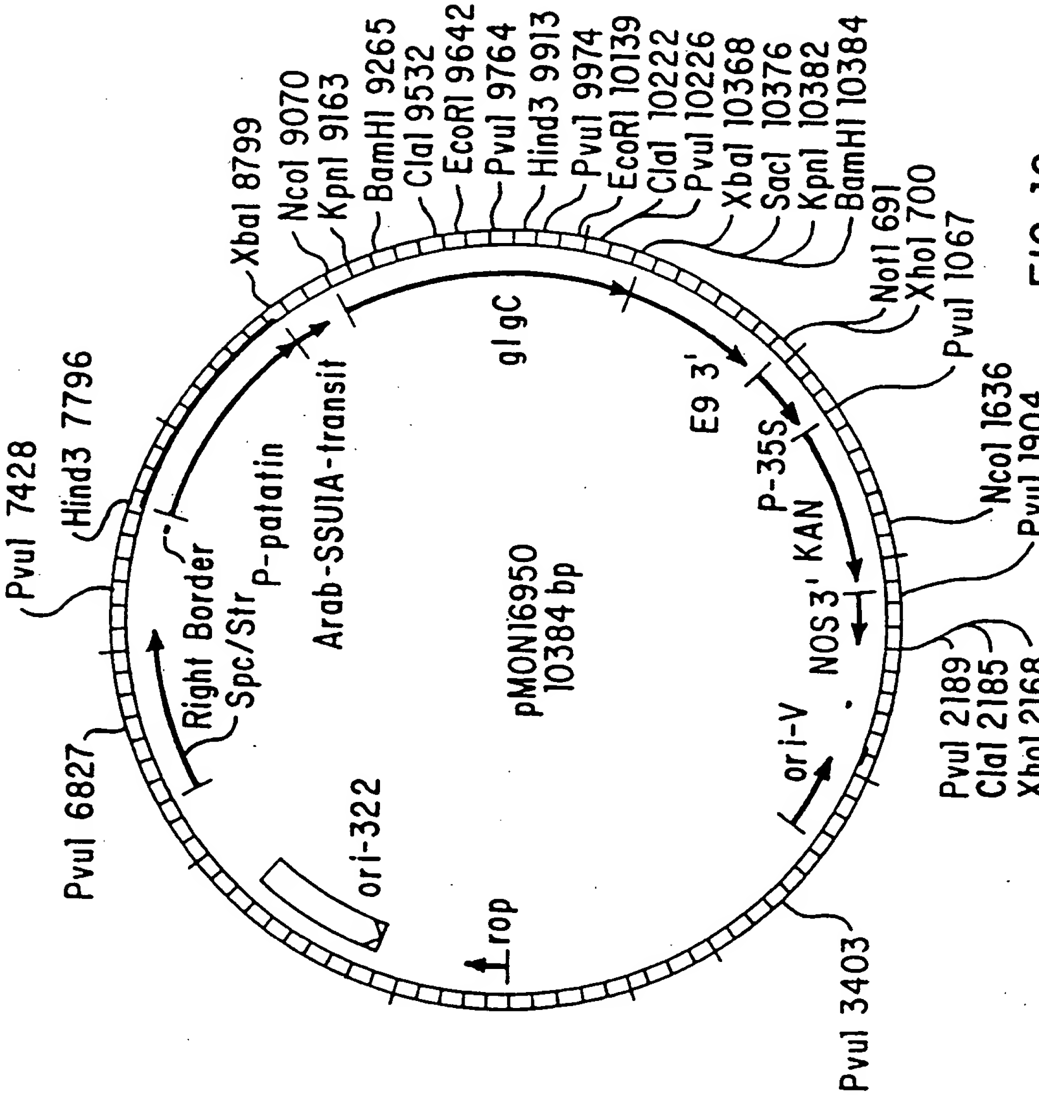

pMON16950
10384 bp
Xbal 8799
Ncol 9070
Kpnl 9163
BamHI 9265
Clal 9532
EcoRI 9642
Pvul 9764
Hind3 9913
Pvul 9974
EcoRI 10139
Clal 10222
Pvul 10226
Xbal 10368
Sacl 10376
Kpnl 10382
BamHI 10384
Notl 691
Xhol 700
Pvul 1067
Ncol 1636
Pvul 1904
Pvul 2189
Clal 2185
Xhol 2168
Pvul 3403
Pvul 6827
Pvul 7428
Hind3 7796
glgC
E9 3'
P-35S
KAN
NOS 3'
ori-V
rop
ori-322
Right Border
Spc/Str
P-patatin
Arab-SSU1A-transit

FIG. 10

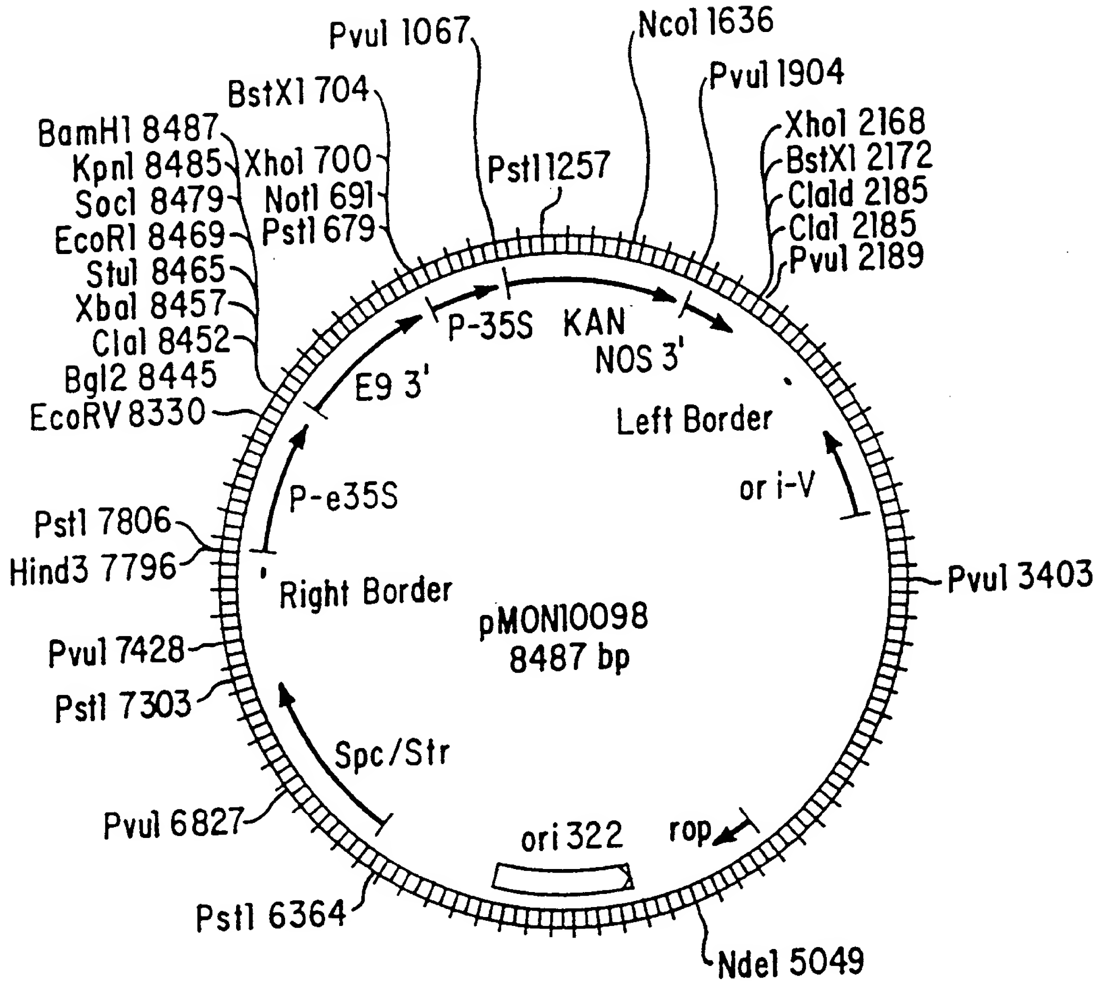

Pvul 1067
Ncol 1636
Pvul 1904
BstX1 704
BamH1 8487
Kpnl 8485
Xhol 700
Socl 8479
Notl 691
EcoR1 8469
Pstl 679
Stul 8465
Xbal 8457
Clal 8452
Bgl2 8445
EcoRV 8330
Pstl 1257
Xhol 2168
BstX1 2172
Clald 2185
Clal 2185
Pvul 2189
P-35S
KAN
NOS 3'
E9 3'
Left Border
or i-V
P-e35S
Pstl 7806
Hind3 7796
Right Border
pMON10098
8487 bp
Pvul 3403
Pvul 7428
Pstl 7303
Spc/Str
Pvul 6827
ori 322
rop
Pstl 6364
Ndel 5049

FIG. 11

BLANK PAGE